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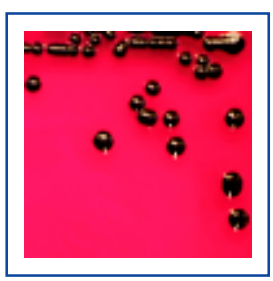
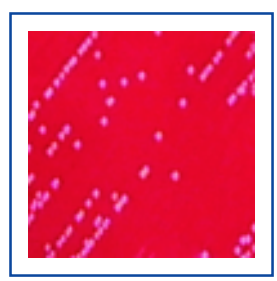
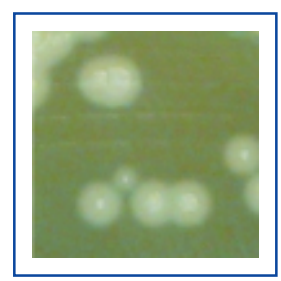
Susanna Lukinmaa

Salmonella enterica, *Listeria monocytogenes* and *Clostridium perfringens*:

Diversity of Human Isolates Studied by Phenotypic and Molecular Methods

Laboratory of Enteric Pathogens, Department of Microbiology
National Public Health Institute
and
Division of General Microbiology, Department of Biosciences
University of Helsinki

Helsinki, Finland
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Diversity of Human Isolates Studied by Phenotypic and Molecular Methods

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Academic dissertation

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Supervised by

Anja Siitonen, M.Sc., Ph.D., Docent
Laboratory of Enteric Pathogens
Department of Microbiology
National Public Health Institute (KTL), Helsinki

Reviewed by

Jorma Hirn, D.V.M., Ph.D., Professor
National Food Agency, Helsinki

Kristina Lindström, M.Sc., Ph.D., Docent
Department of Applied Chemistry and Microbiology
University of Helsinki, Helsinki

Opponent

Sinikka Pelkonen, D.V.M., Ph.D., Professor
National Veterinary and Food Research Institute (EELA),
Kuopio Department, Kuopio

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Kansanterveyslaitos
Mannerheimintie 166
00300 Helsinki
puh (09) 47441
fax (09) 47448238

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsingfors
tel (09) 47441
fax (09) 47448238

National Public Health Institute
Mannerheimintie 166
00300 Helsinki
phone +358-9-47441
fax +358-9-47448238

COVER: *Salmonella enterica* on xylose-lysine-desoxycholate (XLD) agar, *Listeria monocytogenes* on sheep blood agar and *Clostridium perfringens* on egg yolk agar (EYA)

CONTENTS

ABSTRACT

Salmonella enterica, Clostridium perfringens, and *Listeria monocytogenes* are important food-borne pathogens in Finland and worldwide. This study exploited pheno- and genotypic methods to gain detailed information about their diversity. Methods, valuable in tracing the sources of infections and making the surveillance of infections more rapid and easier, were set up.

PFGE was a central method and was very suitable for epidemiological purposes when documented outbreaks and sporadic cases were differentiated. The DNA degradation, sometimes associated with PFGE and making the genotyping of strain impossible, could be prevented by establishing new treatment conditions. Thus, when tris-containing running buffer was changed to HEPES buffer, all previously untypeable *S*. Ohio, *S*. Newport and enterohaemorrhagic *E.coli* non-O157 strains could be typed. However, in the absence of specific phenotypic and epidemiological background data, PFGE was not sufficient to distinguish between strains of *S*. Enteritidis PT1 and PT4, or between pathogenic and normal flora strains of *C. perfringens*. Therefore, before further laboratory typing, for example by PFGE, all *S*. Enteritidis isolates should be phage typed and all *C. perfringens* isolates should be screened for the *cpe* gene which encodes gastrointestinal symptoms-causing enterotoxin.

Automated ribotyping is a very useful tool as the first step method in epidemiological surveys, mainly based its ability to analyse a large number of bacterial isolates in a very short time with minimal human labour. However, the ribotyping of *L. monocytogenes* using restriction by enzyme *Eco*RI brought no extra discrimination to the results of PFGE. Furthermore, without serotyping, the ribotyping with *Eco*RI alone was not discriminative enough in investigating the outbreak associated with butter, caused by the *L. monocytogenes* strain of serotype 3a.

This study confirmed the earlier findings that the *S*. Enteritidis PT1 strain, which had caused outbreaks in Turku and Vieremä from 1991 to 1995, spread from the nearby poultry farm. However, these outbreak strains differed from those associated with the Baltic Countries and Russia. It was also established that three outbreaks caused by *S*. Enteritidis PT4 had different origins. Furthermore, valuable information for future investigations was gained on the distribution of molecular subtypes of the strains imported from the tourist resorts popular among Finns, and on the subtypes of strains isolated from domestic production animals.

The true outbreaks caused by *C. perfringens* in Finland were detected and verified. In two infection clusters, the strains were not the causative agents as earlier suspected. Therefore, the data clearly emphasized the importance of the characterization of the *C. perfringens* isolates in more detail than just identifying them to species level. Furthermore, the PFGE typing or the determination of production of CPE enterotoxin alone may lead to the false negative results. Therefore, the detection of the *cpe* gene by PCR is justified. Also, it is very important to study more than one isolate from the faecal culture of the patient to increase the probability of detecting the cells carrying the *cpe* gene and not just cells being part of the normal flora. Consequently, the recommendation to pick up 10 *C. perfringens* colonies per outbreak has recently been introduced to Finnish routine clinical microbiology laboratories.

The most common *L. monocytogenes* serotypes among human infections in Finland were 1/2a and 4b. The data also

showed that the serotype 1/2a has replaced the serotype 4b. The most prevalent single genotype was PFGE type 1 that has previously been associated with vacuum-packed fish products. The findings also suggested that the outbreak associated with butter, caused by serotype 3a, had already started in 1997. Two interesting findings were also that PFGE type 24 was statistically significantly associated with the male gender and more than one fourth of the industrial findings were never detected causing human infections.

An electronic database library consisting of every different PFGE profile of human *L. monocytogenes* strains was established for continuous, real-time surveillance of *L. monocytogenes* infections and for detecting their potential clustering. At present, the library is in "every day use" in bilateral computer-based networking for comparison of PFGE profiles of *L. monocytogenes* isolates from humans, food and food production environments in collaboration with The National Veterinary and Food Research Institute (EELA).

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-V). In addition, some unpublished data are presented.

I Lukinmaa S, Schildt R, Rinttilä T, Siitonen A. *Salmonella* Enteritidis phage types 1 and 4: pheno- and genotypic epidemiology of recent outbreaks in Finland. *J Clin Microbiol* 1999; 37: 2176-2182.

II Lukinmaa S, Takkunen E, Siitonen A. Molecular epidemiology of food-borne *Clostridium perfringens* related to food-borne outbreaks in Finland from 1984 to 1999. *Appl Environ Microbiol* 2002; 68:3744-3749.

III Lukinmaa S, Aarnisalo K, Suihko M-L, Siitonen A. Diversity of *Listeria monocytogenes* isolates of human and food origins studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. *Clin Microbiol Infect* 2003. In press.

IV Lukinmaa S, Miettinen M, Nakari U-M, Korkeala H, Siitonen A. *Listeria monocytogenes* isolates from invasive infections: variation of sero- and genotypes during an 11-year period in Finland. *J Clin Microbiol* 2003; 41: 1694-1700.

V Koort JMK, Lukinmaa S, Rantala M, Unkila E, Siitonen A. Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis. *J Clin Microbiol* 2002; 40: 3497-3498.

ABBREVIATIONS

AFLP	Amplification fragment length polymorphism
ATCC	American Type Culture Collection
BHI	Brain heart infusion agar
bp	Base pair
CPE	*Clostridium perfringens* enterotoxin
cpe	The *cpe* gene encoding CPE enterotoxin
DI	Discrimination index
DNA	Deoxyribonucleic acid
DT	Definite-type
EAggEC	Enteroaggregative *Escherichia coli*
EELA	Eläinlääkintä- ja elintarviketutkimuslaitos (The National Veterinary and Food Research Institute)
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
GBS	Guillain-Barŕe syndrome
HUS	Haemolytic-uremic syndrome
KTL	Kansanterveyslaitos (National Public Health Institute)
LEP	Laboratory of Enteric Pathogens, National Public Health Institute
LPS	Lipopolysaccharide
kb	Kilobase
NCTC	National Collection of Type Cultures
NIDR	National Infectious Disease Register
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PT	Phage type
ReA	Reactive arthritis
RFLP	Restriction fragment length polymorphism
RH	Reference Helsinki
UPGMA	Unweighted pair-group method with arithmetic averages
VTEC	Verotoxigenic *Escherichia coli*
WHO	World Health Organization

INTRODUCTION

Food-borne diseases are a growing health problem worldwide and Bryan (1982) listed more than 200 different diseases known to be transmitted by food in 1982. Since then, new pathogens such as EHEC O157 and *Cyclospora* have emerged (Oldfield 2001). It has been reported that there are approximately 76 million cases of food-borne diseases in the United States annually (Mead *et al.* 1999) and 1.3 million cases in England and Wales (Adak *et al.* 2002). In Finland, it has been estimated that 500 000 individuals become ill with food-borne disease annually (Niemi *et al.* 1997). Moreover, several thousand individuals might have post infectious complications afterwards (Karmali *et al.* 1983; Keat 1983; Ropper 1992). However, in only a fraction of all these food-borne infections can a certain vehicle be traced and a specific causative agent found. The traditional epidemiological laboratory methods such as biotyping, serotyping and phage typing of isolates, as well as antimicrobial susceptibility testing, do not always give enough information for epidemiological purposes. Thus, the final diagnosis might often be based on just the clinical symptoms of the patients and /or findings in food.

Salmonella enterica, *Clostridium perfringens* and *Listeria monocytogenes* are all important food-borne pathogens from the public health point of view. *Salmonella enterica* has been the leading cause of food-borne illnesses in humans worldwide and the most common serotypes isolated are Enteritidis and Typhimurium (Todd 1997). Furthermore, campylobacters, *C. perfringens*, salmonellas, EHEC O157 and *L. monocytogenes* are among the most important bacterial pathogens in terms of disease burden in many countries (Todd 1997; Mead *et al.* 1999; Adak *et al.* 2002)

In the early 1990s, *S.* Enteritidis caused 15 outbreaks in Finland, and has since then become the most common *Salmonella* serotype among all *Salmonella* strains identified annually (http://www.ktl.fi/ttr). The origin of these outbreaks remained unclear. However, several alternatives could be speculated, for example, the infections were connected to imported foodstuffs, they were secondary infections caused by strains of foreign origin, or there were some hidden reservoirs in Finnish production animals.

When *C. perfringens* has been suspected to be the cause of an outbreak in Finland, the final diagnosis has mainly been based on the clinical symptoms of the patients and/or findings in foods, and the human faecal *C. perfringens* isolates have not generally been studied further.

Serotyping has been a classic phenotypic tool in epidemiological studies of *L. monocytogenes*. However, most of the strains that are important from the public health point of view belong to only three serotypes: 1/2a, 1/2b, or 4b. Furthermore, unlike most other common food-borne infections, listeriosis has a mortality rate of 20-30% (Schlech *et al.* 1983; Fleming *et al.* 1985; Bula *et al.* 1995; Goulet *et al.* 1998; Lyytikäinen *et al.* 2000a; Adak *et al.* 2002) emphasizing the need for timely epidemiological surveillance.

This study was begun in order to gain detailed information on the diversity of food-borne *Salmonella enterica, C. perfringens* and *L. monocytogenes*. To approach this goal various pheno- and genotypic methods were designed and set up.

REVIEW OF THE LITERATURE

1. Food-borne infections and intoxication

Food-borne disease results from ingestion of either food or water contaminated with pathogenic microorganisms, microbial toxins, chemicals, or from consumption of naturally occurring plant or animal toxins (Aucott 1995). In this review of the literature, the main focus will be on pathogenic microorganisms and microbial toxins.

1.1. Terminology

1.1.1. Infections

The food-borne infections are characterized by microbial colonization and multiplication at specific body sites leading to the symptoms. Pathogens, for example, *Campylobacter, Salmonella, Shigella* and *Yersinia* species, as well as *L. monocytogenes,* colonize and invade the gastrointestinal mucosa to reach the underlying tissue. Food-borne infection can also be caused by bacteria producing toxin in the intestinal mucosa of the small intestine, for example, *Bacillus cereus*, *C. perfringens,* enterotoxigenic *E. coli* and *Vibrio cholerae* (Salyers and Whitt 1994).

1.1.2. Intoxication

In food-borne intoxication, the bacteria grow in the food where they produce exotoxins. The exotoxins are ingested and are responsible for the symptoms of food-borne disease without bacterial colonization in the intestine. These toxin-associated food-borne diseases are caused, for example, by *B. cereus, Clostridium botulinum* and *Staphylococcus aureus* (Salyers and Whitt 1994).

1.2. Clinical symptoms

In all cases, infections may not even be suspected to be food-borne; illness may not have prominent gastrointestinal symptoms or it may have a long incubation period. (Table 1).

Most food-borne illnesses are mild with acute gastrointestinal symptoms, such as diarrhoea and vomiting. However, many food-borne pathogens can also cause invasive infection, leading to meningitis or sepsis, and, for example, *L. monocytogenes* can cause miscarriage in pregnant women. Also, various post-infectious complications after food-borne infections have been reported, such as reactive arthritis (ReA) (Keat 1983), haemolytic uremic syndrome (HUS) (Karmali *et al.* 1983) and Guillain-Barŕe syndrome (GBS) (Ropper 1992). Furthermore, an asymptomatic chronic carrier state can follow after food-borne infection (Musher and Rubenstein 1973, Cohen *et al.* 1987).

The varying pathogenic mechanisms by which food-borne microbes produce disease help to explain the variable incubation period observed with different food-borne diseases. In a short incubation period and rapid onset of the illness, the symptoms may be due to preformed toxins produced, for example, by *S. aureus*. When the incubation period is longer, toxins may have been produced in the gastrointestinal tract, for example, by *C. perfringens*. With organisms capable of tissue invasion including *Salmonella, Shigella*, entero-invasive *E. coli, Campylobacter* species and *L. monocytogenes,* the incubation period is even longer (Aucott 1995) (Table 1).

Table 1. Examples of incubation periods of microbes and symptoms they cause. Adapted from Bryan (1995) with modification.

	Incubation period	Predominant sign and symptoms	Examples of microbes
Upper gastrointestinal tract	1-6 h	Nausea, vomiting, retching, diarrhoea, abdominal pain, prostration	*Staphylococcus aureus* and its enterotoxins; *Bacillus cereus* and its emetic toxin
Lower gastrointestinal tract	8-22 h	Abdominal cramps, diarrhoea	*Clostridium perfringens, Bacillus cereus* and its diarrhoeagenic toxin
	12-72 h	Abdominal cramps, diarrhoea, vomiting, fever, chills, malaise	*Salmonella, Campylobacter, Shigella,* diarrhoeagenic *Escherichia coli, Vibrio*
		As above but diarrhoea is bloody	*Escherichia coli* O157:H7, *Shigella*
	1-3 days	Diarrhoea, fever, vomiting, abdominal pain; possible respiratory symptoms	Enteric viruses like rotaviruses, astroviruses, noroviruses
	1-12 days	Profuse watery diarrhoea, abdominal pain, malaise, fever, anorexia, nausea, vomiting	*Cryptosporidium parvum*
	11-70 days	Fever, sepsis, meningitis, diarrhoea, abortion, stillbirth, influenza-like symptoms	*Listeria monocytogenes*
	1-6 week	Mucoid diarrhoea (fatty stools), abdominal pain, weight loss	*Giardia lamblia*
	1 to several weeks	Abdominal pain, diarrhoea, constipation, headache, drowsiness, ulcers, variable - often asymptomatic	*Entamoeba histolytica*

1.3. Risk factors associated with food

Direct contact with faeces, or indirectly through contact between faeces and water, food, utensils, fingers, flies, or the ground, transmits many diarrhoeal diseases. Certain microbes have been in particular associated with specific foods and beverages (Table 2). Furthermore, the composition of the food may affect the possibility of getting food-borne diarrhoea. For example, in high fat food, hydrophobic lipid micelles may protect the smaller amount of bacteria from the action of gastric acidity (D'Aoust *et al.* 2001).

According to the data of food-borne diseases collected worldwide from 46 countries, inadequate cooling and improper cooking was responsible in 44% of cases, contaminated or toxic raw products in 16%, contamination by personnel or equipment in 15%, lack of hygiene in processing, preparing and handling in 10%, and cross-contamination in 4% (Todd 1997).

Table 2. Main vehicles of some food-borne pathogens.

Microbe	Vehicle/Source	Reference
Campylobacter spp.	Poultry Raw milk Water	Friedman *et al.* 2000
C. perfringens	Meat	Todd 1997; Olsen, S. J. *et al.* 2000
E. coli	Cattle meat Water	Meng and Doyle 1998
L. monocytogenes	Cheese Poultry Fish Raw milk	Ryser 1999
Norovirus	Berries Water Seafood	Pönkä *et al.* 1999 Maunula *et al.* 1999 McDonnell *et al.* 1997
Parasites	Water	Dennis *et al.* 1993
S. enterica	Eggs Sprouts Poultry	Todd 1997 Mahon *et al.* 1997; Taormina *et al.* 1999; van Duynhoven *et al.* 2002 Kessel *et al.* 2001
Vibrio spp.	Seafood Water	Kaysner and Hill 1994; Mintz *et al.* 1994
Yersinia spp.	Raw milk Pork	Shayegani *et al.* 1983 Tauxe *et al.* 1987; Ostroff *et al.* 1994; Satterthwaite *et al.* 1999

1.4. Risk factors associated with humans

Large numbers of risk factors that may be associated with diarrhoeal disease have been demonstrated. This includes extremes of age (elderly and children), pregnancy and people with underlying illnesses. People with HIV, for example, are more frequently infected, with *Salmonella, Campylobacter* and *Listeria* (Altekruse *et al.* 1994; Angulo and Swerdlow 1995) than people with no underlying predisposing disease. Furthermore, worldwide movement of goods and people has increased dramatically, at the same time increasing the risk that food-borne outbreaks can spread rapidly at the local, national and international level. Food processing and packaging has also changed over the past 10 years permitting the growth of certain food-borne pathogens (Miettinen *et al.* 1999). Moreover, consumers have changed their food-related behaviour towards eating fresh foods (Niemi *et al.* 1997).

1.5. Factors associated with food-borne pathogens

Different food-borne pathogens have different infective doses that can cause illness (Table 3). The infective dose of food-borne pathogens depends on variables, like conditions of the host, composition of the food (as mentioned above) and the virulence of the infecting strain.

Food-borne pathogens cause infections by a number of different mechanisms (Table 4). Toxin production is one of the major virulence mechanisms. Toxins can be divided into exotoxin and endotoxins. Exotoxins are mainly proteins secreted by a growing bacterial cell into surrounding fluids. Endotoxin is LPS, which is part of the outer membrane of gram-negative bacteria, and the lipid A, which is the toxic part of LPS, is released only when bacteria lyse. Some food-borne microbes produce, for example, neurotoxin, enterotoxin or cytotoxin which are all exotoxins. Neurotoxins are frequently formed on the contaminated food before it has been consumed. These toxins are usually also associated with vomiting and are produced by strains of food-borne *S. aureus* (Salyers and Whitt 1994) and *B. cereus* (Granum 2001). The prototype of enterotoxin is *Vibrio cholerae* toxin. Enterotoxins activate the adenylate cyclace leading to an increase of the local cyclic adenosine monophosphate and, therefore, changing the balance of ionic concentrations. The flow of sodium into tissue decreases and the flow of chloride out of tissue increases, resulting in movement of fluid into the gut lumen (Kaper *et al.* 1994; Salyers and Whitt 1994). Cytotoxins are defined as proteins that kill target cells. They can act intracellularly or form pores in the cell. One example of cytotoxins is Shiga toxin which inhibits cellular protein syntesis and is produced by *S. dysenteriae* serotype 1 (Sandvig 2001). Another example of cytotoxin is listeriolysin O (LLO) which is a pore-forming cytotoxin and is produced by *L. monocytogenes* when a bacterium is escaping a phagocytic vesicle (Vazquez-Boland *et al.* 2001).

Some bacteria, which are not invasive, bind to intestinal cells. However, they do not adhere uniformly over the surface but tend to form clumps and moreover, for example, EAggEC produces Shiga-toxin-like toxin and haemolysin-like toxin (Salyers and Whitt 1994).

Table 3. Infective doses of food-borne pathogens

Pathogen	Infective dose	Reference
Campylobacter spp.	500 -800	Robinson 1981; Black *et al.* 1988
C. perfringens	10^7	Brynestad and Granum 2002
EHEC	<50	Tilden *et al.* 1996
L. monocytogenes	Not known	Rocourt *et al.* 2000
Salmonella spp.	10 - 10^7	D'Aoust *et al.* 2001
Shigella spp.	10 - 100	Sansonetti 2001
S. aureus	10^5 - 10^8	Holmberg and Blake 1984
Vibrio spp.	10^4 - 10^7	Cash *et al.* 1974
Yersinia spp.	10^4 - 10^9	Bottone 1997; Robins-Browne 2001

The invasive organism leaves the intestine and crosses the mucosa to reach underlying tissue. Some food-borne pathogens multiply within the phagosome of the cell, like *Salmonella* (Hansen-Wester and Hensel 2001) or escape from the phagosome and replicate in the cytoplasm, like *Shigella* spp. (Sansonetti 2001) and *L. monocytogenes* (Vazquez-Boland *et al.* 2001). Furthermore, for example, *L. monocytogenes*, followed by an actin-dependent motility process, pass through to the neighbour cells without exposure to extra cellular space.

Table 4. Examples of virulence mechanisms of food-borne pathogens. Adapted with modification from Goodman and Segreti (1999).

Virulence mechanism	Examples of organisms
Neurotoxin	*Staphylococcus aureus* *Bacillus cereus*
Enterotoxin	*Vibrio cholerae* ETEC *Clostridium perfringens* *Bacillus cereus*
Cytotoxin/invasion	*Shigella dysenteria* *Campylobacter jejuni* Amebiasis *Salmonella enterica* *Listeria monocytogenes* *Yersinia enterocolitica*
Adherence	EAggEC

1.6. Burden of diseases

Over 200 diseases are known to be transmitted by food (Bryan 1982). During the past 20 years also several new pathogens have emerged to cause food-borne diseases (Oldfield 2001). Approximately 76 million cases, 325,000 hospitalisations and 5,000 deaths are caused by food-borne diseases in the United States annually (Mead *et al.* 1999). In 2000 in England and Wales, there were 1.3 million cases, 20,800 hospitalisations and 480 deaths due to indigenous food-borne diseases (Adak *et al.* 2002). C*ampylobacter*, *C. perfringens*, *Salmonella*, EHEC O157 and *L. monocytogenes* accounted for the greatest disease burden, such as morbidity, hospital admission and mortality (Adak *et al.* 2002). The leading microbial causes of death have been reported to be *Salmonella, Listeria* and *Toxoplasma* which together account more than 75% of food-borne deaths caused by known pathogens in the Unites States (Mead *et al.* 1999). In Finland, it has been estimated that 500, 000 individuals become ill with food-borne disease annually and the yearly costs are 84 million euros (Niemi *et al.* 1997). In the United States, it has also been reported that unknown agents account for approximately 81% of food-borne illnesses and hospitalisations and 6% of deaths (Mead *et al.* 1999).

Usually, the food-borne illness is self-limited gastrointestinal disease. This is one reason why some food-borne illnesses are under-diagnosed and unreported (Mead *et al.* 1999). WHO has estimated that in industrialized countries, less than 10% of food-borne illnesses are reported, whereas in developing countries the figure is probably less than 1%. Some sick individuals might have mild symptoms and do not seek medical care, many clinicians do not routinely obtain stool cultures from patients, and not all laboratories report isolates to central public health laboratories (Slutsker *et al.* 1998). Alternatively, laboratories do not routinely test stool specimens for every possible food-borne pathogen or they do not have the necessary typing methods to detect the pathogen.

However, the economic cost of diarrhoeal diseases and post infectious complications is high. It may include physicians' visits, medications, hospitalisations and, in-directly, also lost productivity of the sick person through lost working days. In the United States in California, from 1990 to 1999, the estimated 10-year hospitalisation costs for Salmonella were $200 million (Trevejo *et al.* 2003). Hospitalisation rates differed markedly by pathogen. It has been reported that with *Listeria* infections 88% of patients required hospitalisation, *E.coli* O157 37%, *Yersinia* 36%, *Vibrio* 25%, *Salmonella* 22%, *Shigella* 14% and with *Campylobacter* 11% (Kennedy *et al.* 2000).

In Denmark, out of 49, 000 patients studied, the relative mortality with 27, 000 patients infected with *Salmonella, Campylobacter, Yersinia* or *Shigella* was 3.1 times higher than in controls (Helms *et al.* 2003).

1.7. Prevention

Continuous real-time public health surveillance for food-borne infections is critical to improve prevention. Surveillance can allow comparison with historical data helping to characterize trends in occurrences of microbes and identify the outbreaks at an early stage (Mahon *et al.* 1997; Lyytikäinen *et al.* 2000a). Surveillance data also provides information about the food items carrying a high risk of transmitting food-borne pathogens. With this information, patient education can be carried out through published recommendations. Furthermore, industrial hygiene by optimal preparation

of food items, elimination of the organism from food and more frequent screening for the microbes is necessary.

1.8. Surveillance in Finland

Food-borne outbreaks have been under surveillance in Finland since 1975 (Hirn *et al.* 1992). Since 1994, clinical microbiology laboratories have been obligated to report certain microbiological findings including several food-borne pathogens to the National Infectious Disease Registry (NIDR). In 1997, the surveillance of food-borne outbreaks was enhanced and the notification system of suspected food- or waterborne outbreaks was created. Every suspected outbreak must be notified to National Public Health Institute (KTL) and the final reports sent to the National Food Agency that maintains the national register of food-borne outbreaks. The data concerning situation of food-borne pathogens in a 5-year period are presented in Figure 1.

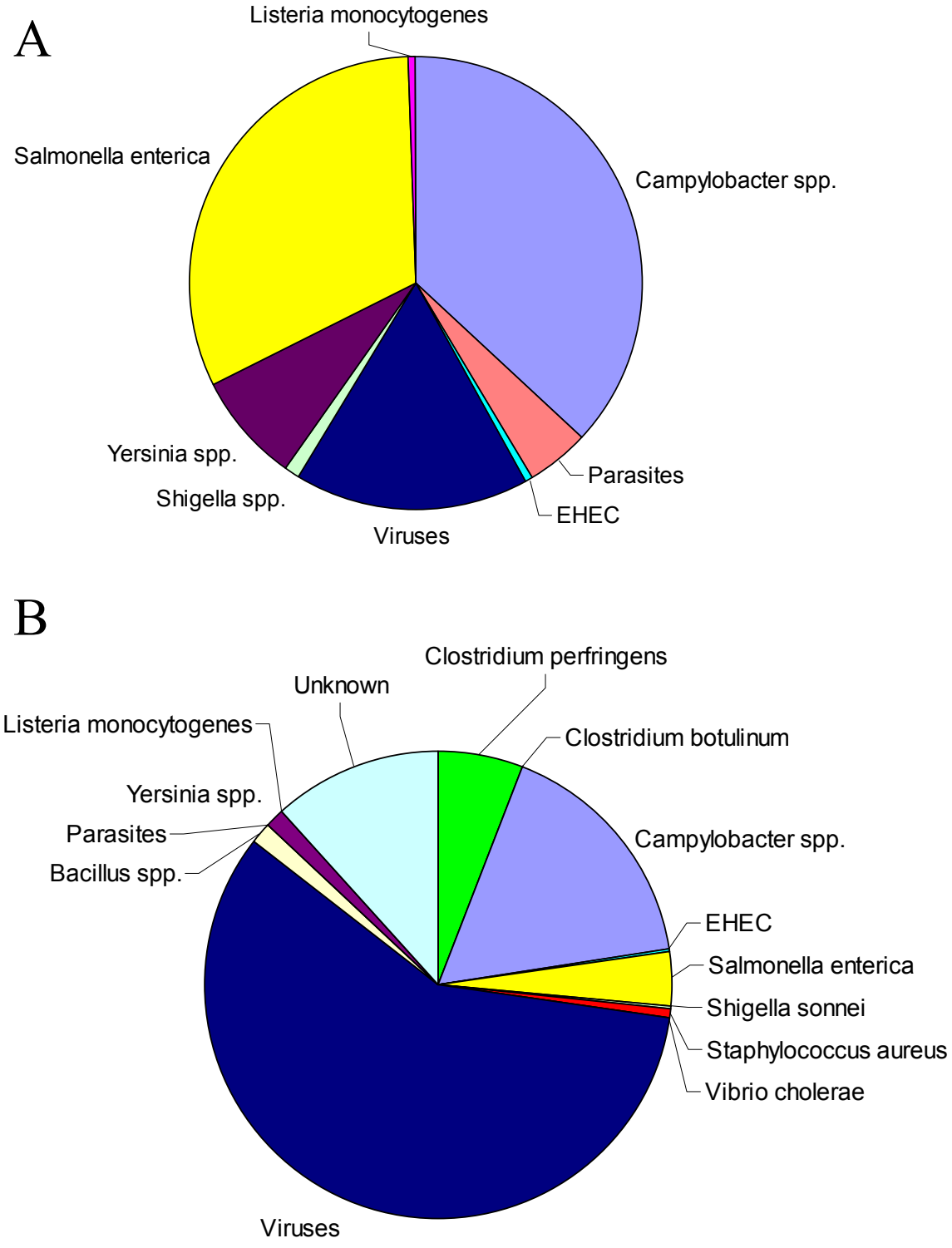
2. Common food-borne pathogens

2.1. Bacteria

Bacteria are the most common agents which cause recognised food-borne microbial diseases. In Finland, about one third of the epidemics were caused by bacteria from 1995 to 2001 (Figure 1B) according to the data recorded in the national food poisoning register kept by the National Food Administration (Kukkula 1998; Hatakka and Wihlman 1999; Hatakka and Halonen 2000; Hatakka *et al.* 2001; Hatakka *et al.* 2002). According to the reports from microbial laboratories to NIDR, the two most common food-borne pathogens in Finland were *Salmonella enterica* and *Campylobacter* spp., accounting for 69% of all reported cases from 1995 to 2001 (Figure 1A). In England and Wales, 45% of cases were caused by bacteria, 0.4% by parasites, 6.3 % by viruses and 48% by unknown agents (Adak *et al.* 2002). In the United States, however, it has been estimated that 80% of food-borne illnesses each year are due to viruses, 13% due to bacteria and 7% due to parasites (Mead *et al.* 1999).

Recently, *S. enterica, V. cholerae*, EHEC O157:H7, *Campylobacter* spp., *L. monocytogenes, Cryptosporidium parvum* and multi-drug resistant pathogens have been referred to as emerging pathogens (Oldfield 2001; Schlundt 2001). Emerging diseases are infections which have newly appeared in a population or already existed but are rapidly increasing in incidence or geographic range (Morse 1995). According to another definition, emerging pathogens have recently increased or are likely to increase within two decades (Altekruse and Swerdlow 1996).

Figure 1. Proportions of food-borne pathogens reported to the National Public Health Institute (KTL) (A) (http://www.ktl.fi/ttr) and number of cases in outbreaks caused by food-borne pathogens reported to the National Food Agency (B) from 1995 to 2001. (Kukkula 1998; Hatakka and Wihlman 1999; Hatakka and Halonen 2000; Hatakka *et al.* 2001; Hatakka *et al.* 2002).



2.1.1. *Salmonella enterica*

The genus *Salmonella* contains two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*, and the first of them (*S. enterica* spp. *enterica*) contains almost all important pathogenic serotypes. According to the O (somatic) antigens, H (flagellar) antigens and Vi (capsular) antigens *Salmonella* strains can be divided into 2501 serotypes (Popoff 2001). Some of the serotypes can be further divided into phage types (PTs) based on host specificity of the bacteriophage. In a global survey of 104 countries, three serotypes, *S.* Enteritidis, *S.* Typhimurium and *S.* Typhi accounted for 76.1% of all isolates reported in 1995 (Herikstad *et al.* 2002).

Salmonella is an intracellular pathogen and can lead to several clinical conditions. The most common disease is self-limiting gastroenteritis. Less than 5% of all patients can develop potentially fatal septicemia when *Salmonella* enters the bloodstream (Pegues *et al.* 1995). Furthermore, *S.* Typhi and *S.* Paratyphi cause typhoid and paratyphoid fever that are serious septic diseases. *Salmonella* infections can also be followed by symptoms of reactive arthritis (ReA) usually after 1 to 3 weeks of infection (Keat 1983; Hannu 2002). ReA has been strongly associated with HLA-B27 histocompatibility antigen (Aho *et al.* 1973) and it has been reported to occur in 1-15% of the patients with salmonellosis (Samuel *et al.* 1995; Mattila *et al.* 1998; Hannu 2002). In Finland, the occurrence of ReA has recently been investigated after three *Salmonella* outbreaks caused by serotype 4,5,12:b:- (Mattila *et al.* 1994), serotype Bovismorbificans (Mattila *et al.* 1998) and serotype Typhimurium (Hannu *et al.* 2002). These studies have shown that the frequency of ReA in Finnish patients has been around 10%.

Salmonella strains can contain a wide variety of plasmids which encode virulence factors and antimicrobial resistance (Gulig *et al.* 1993; Bebora 1997; Rotger and Casadesus 1999) and, therefore, play an important role in pathogenicity. Salmonella virulence plasmids are heterogeneous in size, 50 to 90 kb. However, all share a 7.8 kb region, *spv* (*Salmonella* plasmid virulence), required to confer the virulence phenotype (Gulig *et al.* 1993; Guiney *et al.* 1994).

***S.* Enteritidis** is, according to the World Health Organization (WHO), the most common serotype in many countries (Rodrigue *et al.* 1990; Herikstad *et al.* 2002). The occurrence of the phage types of *S.* Enteritidis in different geographical areas has varied: PT1 has been common in Baltic countries and Russia (Hasenson *et al.* 1992), PT4 in Western European countries (Humphrey *et al.* 1989; Fantasia and Filetici 1994 Schroeter *et al.* 1994; Grimont *et al.* 1999; Tschape *et al.* 1999; Wall and Ward 1999; van de Giessen *et al.* 1999), Japan (Kusunoki *et al.* 1997; Ejidokun *et al.* 2000) and Brazil (Irino *et al.* 1996) and PT 8 and PT 13 have been frequently found in the United States (Hickman-Brenner *et al.* 1991), and PT8 also in Canada (Clark *et al.* 2003). The predominance of certain phenotypes within certain geographical areas makes the effectiveness of phage typing in epidemiological studies limited. Also, inter-conversion among PTs is possible (Threlfall and Chart 1993; Threlfall *et al.* 1993; Powell *et al.* 1995; Rankin and Platt 1995; Brown *et al.* 1999). These PT conversions have been shown to be related to the loss of the ability to express long chain lipopolysaccharide (LPS), like the conversion of highly virulent strains of PT4 to strains of PT7 (Chart *et al.* 1989a). Furthermore, plasmid acquisition has been shown to be connected to these conversions, like the conversion of PT4 to PT24 when acquisition of an incN plasmid

has modified the lytic pattern of *S.* Enteritidis (Frost *et al.* 1989). Phage conversions from PTs 1, 4, 8 to PTs 21, 6 and 13a, respectively, have also been reported (Brown *et al.* 1999).

In epidemiological and environmental studies worldwide, the main sources of *S.* Enteritidis, have been poultry and eggs (Mishu *et al.* 1994; Carraminana *et al.* 1997; Todd 1997; Kessel *et al.* 2001). In Spain, 80% of cold-stored liver in a poultry slaughterhouse was contaminated with *Salmonella* strains, mostly with *S.* Enteritidis (77.6%) (Carraminana *et al.* 1997). In Finland, most of the *S.* Enteritidis infections have been imported from abroad (Figure 2). However, 16 outbreaks occurred from 1991 to 2003 in different parts of Finland; 9, 4 and 3 outbreaks were caused by *S.* Enteritidis PT1, *S.* Enteritidis PT4 and other PTs, respectively.

S. Enteritidis has remained sensitive to most antibiotics (Threlfall *et al.* 2000). However, recently some countries have reported increasing quinolone nalidixic acid resistance in *S.* Enteritidis strains (Molbak and Neimann 2002).

***S.* Typhimurium** is the second most common serotype after *S.* Enteritidis in many countries (Herikstad *et al.* 2002) and resistance to a wide range of antimicrobial agents is common (Threlfall *et al.* 2000). *S.* Typhimurium definitive phage type 104 (DT 104) is one of the emerging multi-resistant phage types. It has spread rapidly in many countries, including England and Wales (Threlfall 2002), Germany, Austria (Prager *et al.* 1999), Canada and the United States (Glynn *et al.* 1998; Swartz 2002). In these countries, the strains are usually resistant to at least five drugs: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su) and tetracycline (T) (resistance type ACSSuT) (Threlfall 2002). Recently, additional resistance to other antimicrobials, including trimethoprim and ciprofloxacin, has been recognised (Threlfall 2002). Over 15% of the multiresistant *S.* Typhimurium DT 104 have been reported to be associated with cases of septisemia in the United States (Miller 1997). In Finland, *S.* Typhimurium is the most common domestic serotype (Figure 3). The first multiresistent *S.* Typhimurium DT 104 was isolated from cattle in Finland in 1995 (Anonymous 2001), however, the level of findings has remained low. The cases of domestic human *S.* Typhimurium DT 104 infections have been less than 20 annually (Anonymous 2001; Anonymous 2003).

The main sources of *S.* Typhimurium have been reported to be cattle and pigs but it is also found in poultry, sheep and goats (Wall *et al.* 1995; Rabsch *et al.* 2002). Traditionally, *S.* Typhimurium has been thought of as the broad-host-range serotype since it is frequently associated with diseases in numerous species, including humans, livestock, domestic fowl, rodents and birds. However, Rabsch *et al.* (2002) presented the alternate view that some variants of the serotype Typhimurium have a very narrow host range. According to their study, the variants that are associated, for example, with disease in pigeons may be considered highly host adapted.

In addition to Enteritidis and Typhimurium serotypes, other common nontyphoidal *Salmonella* serotypes identified in Finnish patients have been *S.* Agona, *S.* Infantis, *S.* Hadar, *S.* Virchow, *S.* Newport, *S.* Stanley and *S.* Panama from 1995 to 2001 (http://www.ktl.fi/ttr). *S.* Hadar, *S.* Virchow, and *S.* Infantis have been reported to be resistant to a wide range of antimicrobial agents (Threlfall *et al.* 2000).

Figure 2. Number of all salmonella infections, and number of domestic and foreign *S.* Enteritidis infections in Finland between 1965 and 2001 (Statistics of the Laboratory of Enteric Pathogens (LEP), National Public Health Institute, Helsinki and http://www.ktl.fi/ttr)

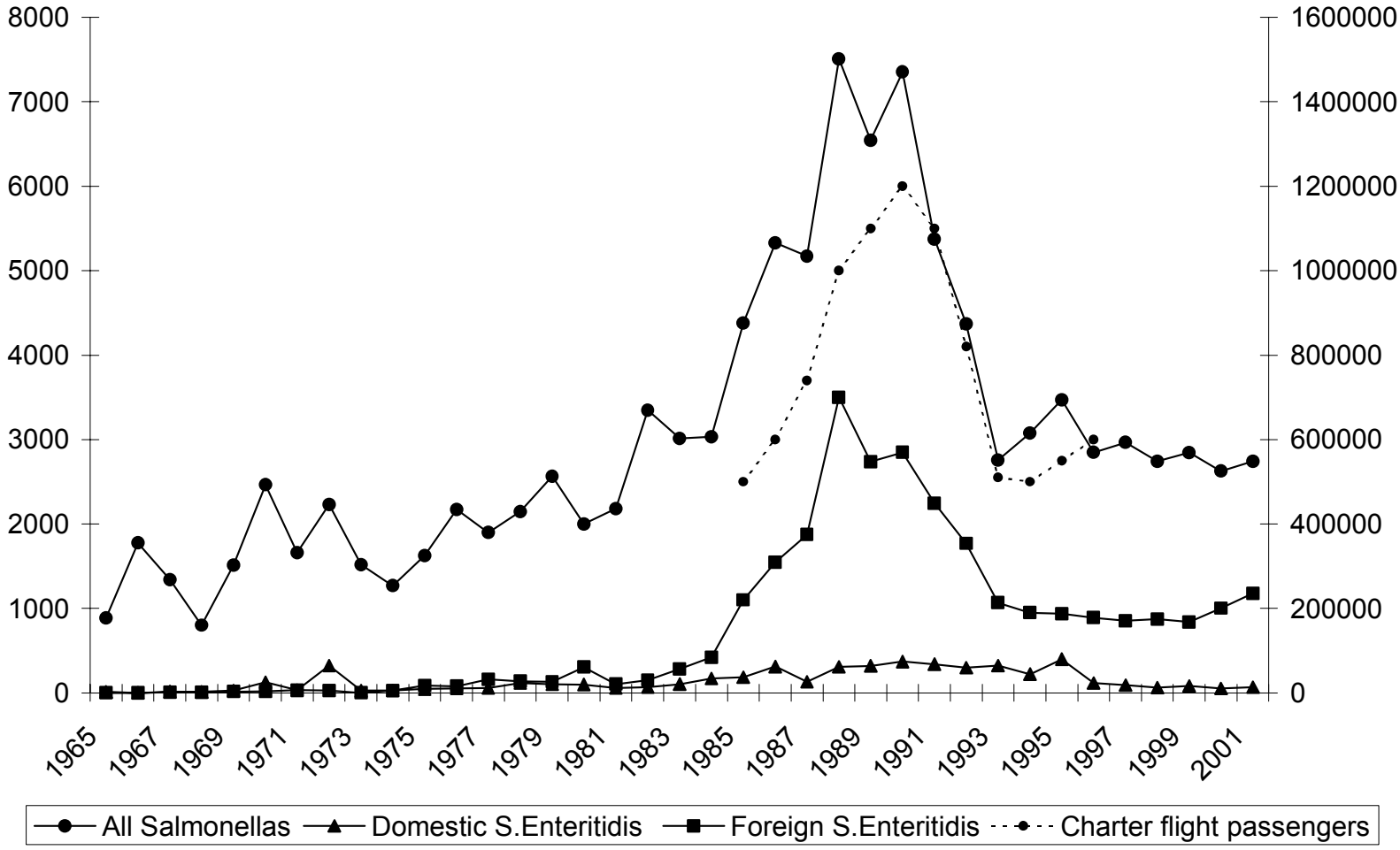
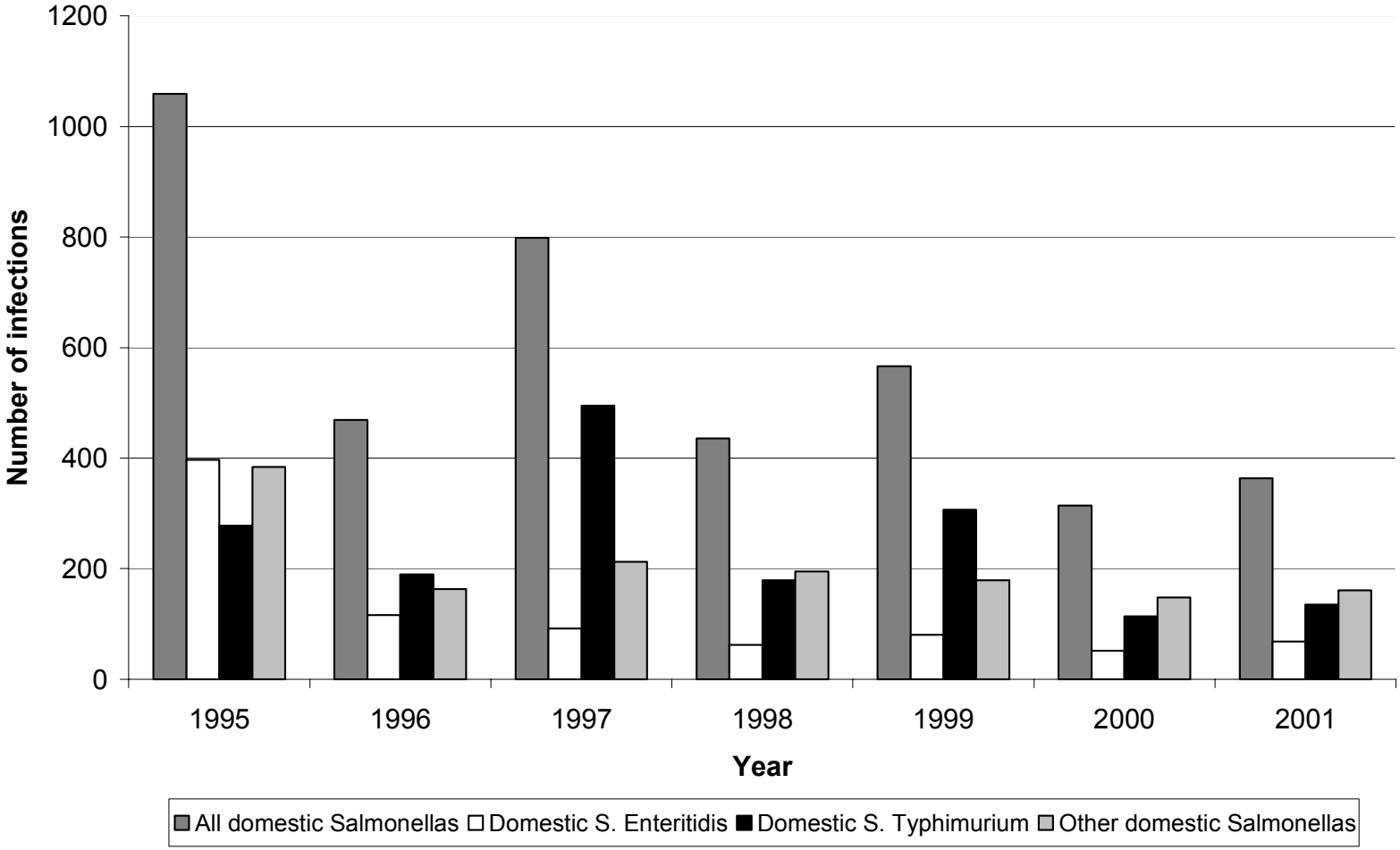


Figure 3. Number of all domestic *Salmonella* infections, and number of all domestic *S.* Enteritidis, *S.* Typhimurium and other *Salmonella* serotype infections in Finland between 1995 and 2001 (http://www.ktl.fi/ttr).



2.1.2. *Clostridium perfringens*

The genus *Clostridium* is extremely heterogeneous: it includes 83-130 validly described species. *C. perfringens* and *C. botulinum* are medically important species of this genus. *C. botulinum* has its natural habitat in soil and aquatic sediments and can be fatal, therefore, it is an epi-demiologically important pathogen (Hielm 1999). However, outbreaks caused by *C. botulinum* are very rare in Finland. Also, *C. perfringens* is widely distributed in the environment and is part of the normal flora of the intestinal tract of humans and animals (Saito 1990). *C. perfringens* is classified into five types (A to E) according to the enterotoxin produced (McDonel 1986; Sarker *et al.* 2000). The enterotoxin (CPE) produced by type A *C. perfringens* is responsible for the symptoms of food poisoning (Skjelkvale and Uemura 1977; Larsson and Borriello 1988; Sarker *et al.* 1999). The CPE is produced during sporulation of the organism in the small intestine after ingestion of *C. perfringens* cells.

The disease is self-limiting, and symptoms disappear within 1 or 2 days. Therefore, most people do not come into contact with health authorities and the number of cases is probably greatly underestimated. However, *C. perfringens* is still registered as one of the most common food-borne diseases (Mead *et al.* 1999; McClane 2001a; Adak *et al.* 2002). Recent estimates indicate that 250,000 cases actually occur in the United States each year, causing an average of seven deaths per year (Mead *et al.* 1999) and the economic cost has been estimated to exceed $120 million (Todd 1989).

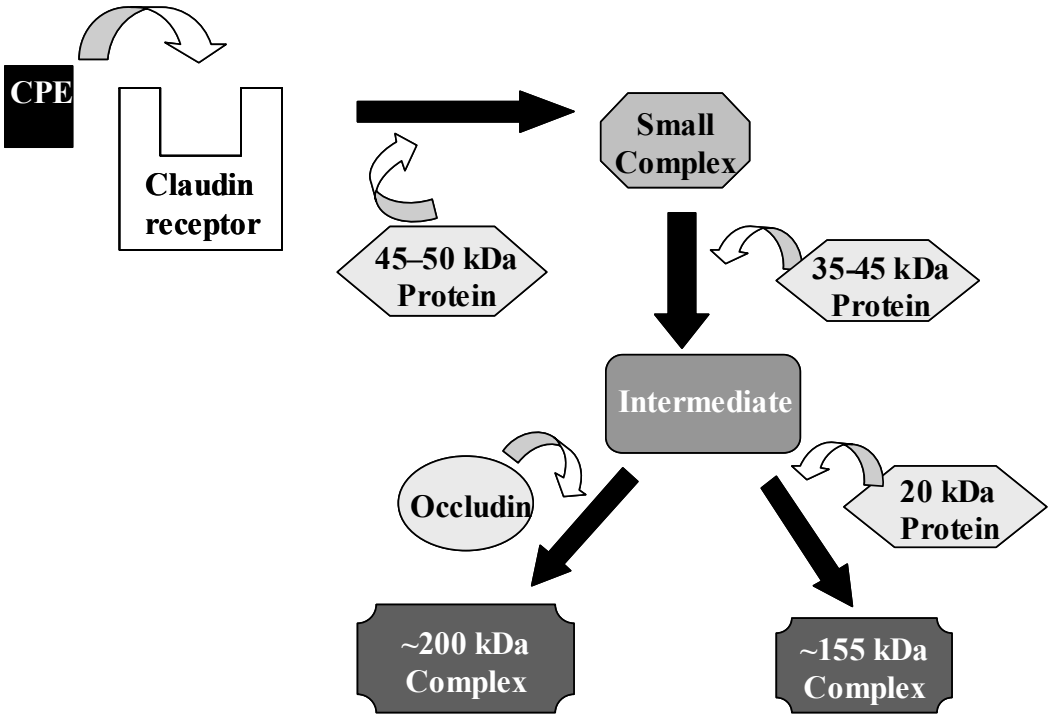
In addition to the ability to produce CPE enterotoxin, *C. perfringens* has also the ability to multiply rapidly in food and form heat-stable spores (McClane 2001a). Outbreaks caused by *C. perfringens* are usually large and most cases are reported from restaurants, hospitals and homes for the elderly. In the United States (Olsen, S. J. *et al.* 2000) and in France the most common vehicle has been meat (Todd 1997). Also in Finland, most of the outbreaks have occurred in mass events from 1997 to 2001 and the most common causes have been meat or meat products (Table 5).

The CPE enterotoxin begins its activity when it binds to intestinal receptor(s). The number and identity of these receptors are still under investigation. However, in some models, two related 22 kDa proteins have been shown to be functional CPE receptors (Katahira *et al.* 1997a; Katahira *et al.* 1997b). These receptors are members of the claudin family of the tight junction proteins (Furuse *et al.* 1998; Morita *et al.* 1999). It has been observed in many studies that CPE forms three complexes, small, intermediate and large (Figure 4). The small complex is formed when CPE binds to the receptor and to a 45 to 50-kDa eukaryotic protein. This happens at 4ºC. When the temperature increases to 37ºC, an intermediate (~135 kDa) and two large (~155 kDa and ~200 kDa) complexes are rapidly formed from the small complex (Singh *et al.* 2000; McClane 2001b). The approximately 200 kDa large complex contains the intermediate and the occludin which is a ~65 kDa tight junction protein (Singh *et al.* 2000). The ~155 kDa large complex has been shown to induce membrane permeability effects (Singh *et al.* 2000).

Table 5. Vehicles and dining location of *C. perfringens* outbreaks in Finland from 1997 to 2001 (Kukkula 1998; Hatakka and Wihlman 1999; Hatakka and Halonen 2000; Hatakka *et al.* 2001; Hatakka *et al.* 2002).

	Vehicles			Dining location								
Year	Meat or meat products	Fish or fish products	Vegetables	Staff canteen	Restaurant/hotel	Day-care centre	Hospital/ home for the elderly	School	Camping centre	Event	Home	Other
2001	1								1			
2000	5	2		2	1				1	2	1	
1999	9	1		3	2	1	1			2	1	1
1998	5	1	1	1	1					3	3	
1997	7	1			2			1		4	1	
Total	27	5	1	6	6	1	1	1	2	11	6	1

Figure 4. A current model of action mechanism of CPE enterotoxin. Adapted from McClane (2001a) with modification.



Recent studies indicate that in most *C. perfringens* strains associated with food poisoning, the *cpe* gene has a chromosomal location, whereas in non-food-borne gastrointestinal diseases the *cpe* gene is located on a plasmid (Cornillot *et al.* 1995; Katayama *et al.* 1996; Collie and McClane 1998). Furthermore, the *cpe* plasmid of at least some isolates from non-food-borne gastrointestinal diseases can transfer via conjungation (Brynestad *et al.* 2001). It has also been proposed that the chromosomal *cpe* gene is located on a transposon (Brynestad *et al.* 1997; Brynestad and Granum 1999). Thus, both the chromosomal *cpe* gene and the plasmid *cpe* gene may be present on mobile elements.

Another type of *C. perfringens* food poisoning is Type C human necrotic enteritis (Granum 1990). However, it is rare in the industrialized world and has not been reported in Europe during the last decade (Brynestad and Granum 2002).

2.1.3. Listeria monocytogenes

The genus *Listeria* contains six species*: L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*. In addition to *L. monocytogenes*, *L. ivanovii*, *L. welshimeri* (Andre and Genicot 1987) and *L. seeligeri* (Rocourt *et al.* 1986) have been implicated in human listeriosis, although, those cases have been very rare. In practice, the only important human pathogen is *L. monocytogenes*. It was first described by Murray *et al.* (1926) and is commonly found in soil, water, and on plant material (Fenlon 1999).

Strains of *Listeria* species are divided into serotypes based on somatic (O) and flagellar (H) antigens (Seeliger and Höhne 1979). Serotyping identifies 13 serotypes of *L. monocytogenes*. However, most of the human cases have been caused by only three serotypes, 1/2a, 1/2b and 4b (Farber and Peterkin 1991; Low *et al.* 1993).

L. monocytogenes has unique features, such as the ability to tolerate adverse environmental conditions. It is able to grow in a wide range of temperatures (0 to 45 ºC) (Junttila *et al.* 1988), pH values 4.3 to 9.0 (Farber *et al.* 1989), and in high salt concentrations (10% NaCl) (Sorrells and Enigl 1990; Lou and Yousef 1999). It not only survives, but can also grow at refrigerator temperatures of 4 ºC. Its ability to survive for long periods under adverse environmental conditions and to colonize, multiply and persist on processing equipment makes it a particular threat to the food industry.

L. monocytogenes causes a serious type of food-borne infection associated with a mortality of about 20-30% (Schlech *et al.* 1983; Fleming *et al.* 1985; Bula *et al.* 1995; Goulet *et al.* 1998; Lyytikäinen *et al.* 2000a). It has been a well-known cause of meningitis and other invasive infections in immuno-compromised patients for a long time. However, only 20 years ago it has been shown to be predominantly food-borne (Schlech *et al.* 1983), causing infection in certain well-defined high-risk groups, including pregnant women, neonates and immuno-compromised adults (Farber and Peterkin 1991; Schuchat *et al.* 1992).

L. monocytogenes is an invasive organism that crosses the intestinal mucosa to reach underlying tissues. It infects and multiplies within host cells in the cytoplasm spreading to the neighbouring cells without exposure to extracellular space (Vazquez-Boland *et al.* 2001) thus evading immune defenses such as antibodies, complement or neutrophils (Southwick and Purich 1996).

Several outbreaks of listeriosis have been described worldwide and most of them have been caused by serotype 4b (Table 6). However, most cases of the human listeriosis occur sporadically (Swaminathan *et al.* 2001) and are most commonly caused by serotype 1/2a (Gerner-Smidt *et al.* 1995; McLauchlin and Newton 1995; Loncarevic *et al.* 1998; Pak *et al.* 2002). In Finland, the rare *L. monocytogenes* serotype 3a has also caused an outbreak in 1998 to 1999. In this outbreak, 25 outbreak-associated patients were identified; the outbreak strain was isolated from butter and the mortality rate was 24% (Lyytikäinen *et al.* 2000a). Furthermore, two other listeriosis, one invasive and one non-invasive, has been connected to a specific source in Finland; salted mushrooms in 1989 (Junttila and Brander 1989) and gastrointestinal non-invasive infection when the patient had no known underlying diseases was caused by rainbow trout in 1998 (Miettinen *et al.* 1999).

This food-borne febrile gastrointestinal syndrome in healthy persons has also been reported in other countries (Riedo *et al.* 1994; Salamina *et al.* 1996; Dalton *et al.* 1997; Heitmann *et al.* 1997; Aureli *et al.* 2000; Hof 2001; Frye *et al.* 2002). The infectious dose of *L .monocytogenes* in these gastrointestinal non-invasive infections is suspected to be higher than in invasive infections. These gastroenteritis cases are also often undocumented, as the disease does not progress to invasive disease, and in gastroenteritis cases *L. monocytogenes* is not commonly included in routine testing procedures. Furthermore, up to 6% of healthy individuals may be carriers of *L. monocytogenes* (Bojsen-Moller 1972; Muller 1990; MacGowan *et al.* 1991; Mascola *et al.* 1992; Schuchat *et al.* 1993; Slutsker and Schuchat 1999).

Table 6. Reported *L. monocytogenes* outbreaks from1979 to 2001.

Country	Year	Vehicle of infection	Serotype	No. of cases	Infection type	Mortality %	Reference
Canada	1979-1981	Coleslaw	4b	41	Invasive	-	Schlech *et al.* 1983
Switzerland	1983	Soft cheese	4b	57	Invasive	32	Bula *et al.* 1995
United States	1983	Pasteurised milk	4b	49	Invasive	29	Fleming *et al.* 1985
United States	1985	Mexican-style cheese	4b	142	Invasive	34	Linnan *et al.* 1988
United States	1986-1987	Unknown	-[a]	36	Invasive	44	Schwartz, B. *et al.* 1989
United Kingdom	1987-1989	Paté	4b	-	-	-	McLauchlin *et al.* 1991
Finland	1989	Salted mushrooms	4b	1	Invasive	0	Junttila and Brander 1989
United States	1989	Shrimp	4b	2	Non-invasive[b]?	1 fetal demise	Riedo *et al.* 1994
France	1993	Rillettes	4b	38	Invasive	32	Goulet *et al.* 1998
Italy	1993	Rice salad	1/2b	39	Non-invasive[b]	0	Salamina *et al.* 1996
United States	1994	Chocolate milk	1/2b	45	Non-invasive[b]	0	Dalton *et al.* 1997
France	1995	Raw-milk soft cheese	-	20	Invasive	20	Goulet *et al.* 1995
Italy	1997	Sweet corn	4b	1566	Non-invasive[b]	0	Aureli *et al.* 2000
Denmark	1997	Unknown	4	3	Non-invasive[b]	0	Heitmann *et al.* 1997
Finland	1998	Rainbow trout	1/2a	5	Non-invasive[b]	0	Miettinen *et al.* 1999
Finland	1998-1999	Butter	3a	25	Invasive	24	Lyytikäinen *et al.* 2000a
United States	1998-1999	Hot dog/deli meats	4b	50	Invasive?	16	Anonymous 1999
France	1999-2000	Rillettes	4b	10	Invasive	10	de Valk *et al.* 2001
France	1999-2000	Jellied pork tongue	4b	32	Invasive	31	de Valk *et al.* 2001
United States	2001	Sliced turkey	1/2a	16	Non-invasive[b]	0	Frye *et al.* 2002

[a] multiple strains (serotypes 4b, 1/2b, 1/2a, 3b)
[b] gastroenteritis and fever

2.2. Other bacteria

Bacillus cereus

Bacillus cereus belongs to the genus *Bacillus*, which is very heterogeneous. *B. cereus* is widespread in nature (Drobniewski 1993) and can easily spread to foods (Granum 2001). *B. cereus* causes two different types of gastrointestinal infections: the diarrheal type caused by enterotoxin(s) and the emetic type caused by a toxin called cereulide (Granum 2001). The enterotoxin is produced during the vegetative growth of *B. cereus* in the small intestine and the emetic toxin is produced by cells growing in food (Kotiranta *et al.* 2000). Like *Clostridium* spp., *B. cereus* is also a sporeforming pathogen and the spores can survive, for example, milk pasteurisation causing problems for the food industry (Andersson *et al.* 1995). The vehicles most frequently associated with diarrheal illness are vegetables, meat and milk products, whereas emetic illness has been associated with rice and pasta (Granum 2001).

The food-borne infections usually have mild symptoms and last less than 24 hours, and are thus not always diagnosed. Furthermore, *B. cereus* is not a reportable disease, therefore, the number of cases in different countries is probably greatly underestimated (Kotiranta *et al.* 2000). However, the diarrheal type has been reported more often in Europe and North America and the emetic type in Japan (Granum 2001).

***Campylobacter* species**

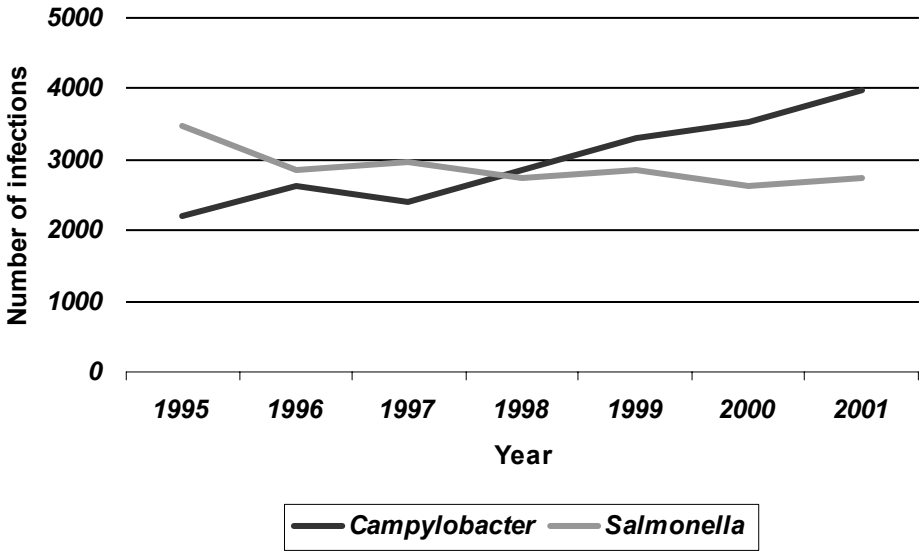
The genus *Campylobacter* contains 14 species, of which *C. jejuni* and *C. coli* are the campylobacters most commonly searched for in diarrhoea. In industrialized countries, *C. jejuni* is the most common cause of *Campylobacter* infections, accounting for approximately 80 to 90% of cases, and *C. coli* for approximately 5 to 10 % of cases (Nachamkin *et al.* 2000).

Campylobacter was first time isolated from human stool and recognized as an important human pathogen in the 1970s (Dekeyser *et al.* 1972; Skirrow 1977). In Finland, the first *Campylobacter* infections were reported in 1978 (Kosunen 1978). In Denmark (http://www.serum.dk), the United Kingdom (Adak *et al.* 2002), the Netherlands (de Wit *et al.* 2001) and the United States (Mead *et al.* 1999), it has been recognized as the most common culture-confirmed food-borne pathogen causing diarrhoea. Also in Finland, the number of *Campylobacter* infections has overtaken (http://www.ktl.fi/ttr) the number of *Salmonella* infections (Figure 5). The majority of infections are sporadic cases. The infection is often seasonal, and targets mostly children and young adults (Friedman *et al.* 2000). Emerging resistance to fluoroquinolones among human *Campylobacter* isolates has also been reported (Reina *et al.* 1994; Piddock 1995; Ruiz *et al.* 1998).

The source, transmission routes and pathogenesis of *C. jejuni* are not fully understood (Ketley 1997). Black *et al.* (1988) have shown that the infectious dose can be as low as 500-800 cells and human infections are usually acquired through the consumption of contaminated food (especially poultry) or water (Blaser 1997). *C. jejuni* has also caused four large waterborne outbreaks in Finland from 1998 to 2001 (http://www.ktl.fi/ttr). All outbreaks were associated with contaminated drinking water (Hänninen *et al.* 2003).

It has been reported that the frequency of ReA has varied from 0.7 to 8% caused by *Campylobacter* (Eastmond *et al.* 1983; Bremell *et al.* 1991). In addition, *Campylobacter* infections have also been implicated in the Guillain-Barŕe syndrome (GBS) which is an autoimmune-mediated disorder of the peripheral nervous system (Ropper 1992).

Figure 5. Number of *Salmonella* and *Campylobacter* infections in Finland from 1995 to 2001 (http://www.ktl.fi/ttr).



Escherichia coli

Of the five *Escherichia* species (*E. blattae*, *E. coli*, *E. fergusonnii*, *E. hermannii* and *E. vulneris*), *E. coli* is the most commonly isolated species from human specimens. Isolates of *E. coli* have been serologically differentiated based on the O (somatic), H (flagella) and K (capsule) antigens. Gastrointestinal infections are caused by five diarrhoeagenic groups: entero-aggregative *E. coli* (EAggEC), entero-haemorrhagic *E. coli* (EHEC), entero-invasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). The classification into these groups is based on virulence factors, mechanisms of pathogenicity, clinical syndromes and distinct O:H serotypes (Bopp *et al.* 1999).

EHEC has emerged as a major public health threat since fewer than 50 viable bacteria have been shown to produce gastrointestinal symptoms (Tilden *et al.* 1996). EHEC O157:H7 was first recognized as a human pathogen in 1982 by Riley *et al.* (1983). It produces two exotoxins called verotoxins (VT), Shiga toxins (Stx) or Shiga-like toxins (SLT), because of the similarity to the Shiga-toxin of *Shigella dysenteria* serotype 1. EHEC organisms are associated with a severe and sometimes fatal condition, haemolytic-uremic syndrome (HUS) (Karmali *et al.* 1983). Approximately 5% of EHEC cases result in HUS and 5% of these cases are fatal (Mead and Griffin 1998).

Outbreaks of EHEC have been associated with eating minced beef, vegetables, drinking apple juice, raw milk, or swimming water contaminated with faeces or person-to-person spread (Parry and Palmer 2000; Terajima *et al.* 2000). In Finland, EHEC caused an outbreak in 1997 which involved 18 culture-confirmed cases and fresh lake water was recognized as the vehicle of EHEC O157:H7 transmission (Paunio *et al.* 1999). The mortality rate was 11%. Also, EHEC O157 infection occurred amongst holidaymakers returning from Fuerteventura, Canary Islands in 1997. Fourteen confirmed cases and one probable were identified among travellers from England (7 cases), Finland (5 cases), Wales (1 case), Sweden (1 case) and

Denmark (1 case) staying in four hotels. The water from a private well was the vehicle of transmission (Pebody *et al.* 1999).

***Shigella* species**

The genus of *Shigella* includes four species, *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. Flagellar and capsular antigens are not produced by *Shigella* strains. Therefore, their antigenic characterization relies only on the properties of their somatic O antigens. *Shigella* causes a disease called dysentery or shigellosis and the stools of patients often contain blood and mucus. *S. sonnei* is the most common and *S. flexneri* the second most common cause of shigellosis in the United States (Lee *et al.* 1991).

Although *Shigella* species stimulate a strong inflammatory reaction, they have developed a range of strategies to escape the host immune response (Zychlinsky *et al.* 1992; Mantis *et al.* 1996). Shigella bacteria are invasive and after cellular entry, bacteria lyse the vacuole and gain access to the cytoplasm, where they divide and spread from cell to cell (Sansonetti 2001). All virulent isolates of *Shigella* carry a large 220 kb virulence plasmid which is essential for invasion. In addition, most of the chromosomal loci associated with *Shigella* virulence are involved in regulation or survival within the host (Sansonetti 2001).

Only a low infectious dose, 10-100 organisms, is required to cause the symptoms (Sansonetti 2001). Therefore, *Shigella* can also easily spread from person-to-person. Shigellosis, especially among young infants, may be fatal. It has also been associated with the subsequent development of haemolytic-uremic syndrome (HUS) in children (Koster *et al.* 1978). According to epidemiological studies, the cause of HUS may be the Shiga toxin produced by *S. dysenteriae* serotype 1 (Lopez *et al.* 1989).

Food-borne outbreaks caused by *Shigella* species have not been associated with any specific vehicle (Lampel and Maurelli 2001). However, outbreaks have occasionally been associated with potato salad, chicken, tossed salad and shellfish. In 1994, in three European countries *S. sonnei* caused outbreaks connected to iceberg lettuce (Kapperud *et al.* 1995). In Finland, *S. sonnei* caused an outbreak in 2001 involving 41 cases (http://www.ktl.fi/ttr).

Staphylococcus aureus

The genus *Staphylococcus* is divided into more than 23 species and subspecies. Nearly all staphylococcal food poisonings are attributed to *S. aureus* (Jablonski and Bohach 2001). It has been estimated that 20 to 50 % of healthy individuals carry *S. aureus* and it has been found in different parts of the human body, such as the nose, colon, vaginal tract and skin (Tranter 1990).

The symptoms of staphylococcal food poisoning are not due to the ingestion of live microorganisms. *S. aureus* causes intoxications and, therefore, the symptoms are produced by ingestion of soluble heat-stable staphylococcal enterotoxin which is preformed in contaminated food (Wieneke *et al.* 1993). Cooking the contaminated food can kill the bacteria, but it will not destroy the heat-stable toxin that has been produced by *S. aureus* (Aucott 1995). The mechanism of the action of staphylococcal enterotoxin is not completely understood.

Humans and animals are the main reservoir of staphylococci. Colonized human carriers provide a good source for dissemination of staphylococci from humans to food. *S. aureus* can also cause mastitis in cattle and contaminate milk before it has been processed (Jablonski and Bohach 2001).

Vibrio

The genus of *Vibrio* contains over 30 species. *V. cholerae, V. mimicus, V. parahaemolyticus, V. vulnificus, V. fluvialis, V. furnissii, V. hollisae* and *V. alginolyticus* are known to be directly food-associated. *Vibrio* species can be found in saltwater or freshwater. The animal reservoir for vibrios is marine invertebrates, particular oysters, crabs and shrimps (Oliver and Kaper 2001; Reidl and Klose 2002). Raw or undercooked clams and oysters, in particular, are associated with the acquisition of *Vibrio* species, leading to diarrhoea.

V. cholerae has originally been divided into serogroup O1 and a group of non-O1 strains (Gardner and Venkatraman 1935). The O1 serogroup has two biotypes, Classical and El Tor, and is the principal cause of cholera (Dalsgaard *et al.* 1997; Yamasaki *et al.* 1997; Bag *et al.* 1998). The new variant, *V. cholerae* O139 (Bengal) has been considered to be the result of gene transfer between O1 serogroup and strains belonging to non-O1 serogroups (Bik *et al.* 1995). This strain has raised serious concern in public health because the disease it causes is clinically indistinguishable from the cholera caused by O1 *V. cholerae*. Studies of the molecular evolution of strains of *V. cholerae* isolated in India, Pakistan, Peru and Thailand have shown that *V. cholerae* O1 undergoes genetic changes relatively frequently (Bik *et al.* 1995; Dalsgaard *et al.* 1997; Yamasaki *et al.* 1997; Bag *et al.* 1998; Dalsgaard *et al.* 1998) and it can spread rapidly (Anonymous 1993; Chongsa-nguan *et al.* 1993; Sheikh *et al.* 1997). However, most of *V. cholerae* strains belong to the serogroup non-O1 which may cause mild gastroenteritis or systemic infections (Saha *et al.* 1996). In Japan, also *V. parahaemolyticus* is a frequent cause of diarrhoea illness and outbreaks (Oliver and Kaper 2001).

In the small intestine, *V. cholerae* adhere to the mucosal surface and start to produce an exotoxin, cholera toxin. The toxin affects the balance of mucosal cells and fluid loss is so significant that a previously healthy person can die within hours of the onset without appropriate fluid replacement therapy (Salyers and Whitt 1994).

***Yersinia* species**

The genus *Yersinia* contains 11 species; *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* are pathogenic for humans. *Yersinia* has been isolated from animals, lake and stream water and a variety of foods (Bottone 1997).

Yersinia has both plasmid-encoded (pYV, plasmid for Yersinia virulence) and chromosomally encoded virulence factors (Bottone 1999). Pigs are a particularly important reservoir. *Yersinia* strains have been isolated from pig tonsils and intestines (Tauxe *et al.* 1987). Fredriksson-Ahomaa (2001) showed that in Finland contaminated pig offal is an important vehicle in the transmission of *Y. enterocolitica* bioserotype 4/O:3 from slaughterhouses to humans. In Finland, *Yersinia* infections have mostly been thought to be domestic ones (Siitonen 2003).

Y. enterocolitica can be divided into 70 serotypes based on the variability of O-antigen (Wauters *et al.* 1991). However, only certain serotypes (mostly O:3, O:9, O:8) of *Y. enterocolitica* cause infections in humans (Bottone 1997). Therefore, the pathogenic potential of a *Y. enterocolitica* isolate should be based on both serotype and biotype (Bottone 1999). In Finland and other Scandinavian countries, Belgium, Canada and Japan, serotype O:3 and O:9 are the most common isolates, while in the Unites States serotype O:8 predominates (Skurnik 1999). Acute *Y. enterocolitica* infection, especially caused by serotypes

O:3 and O:9, can also trigger ReA. It seems that ReA is more frequent in Scandinavian countries where HLA-B27 and serotypes O:3 and O:9 are prevalent (Bottone 1999). Most infections caused by *Y. enterocolitica* occur in children younger than 5 years old (Hoogkamp-Korstanje and Stolk-Engelaar 1995).

In contrast to *Y. enterocolitica*, all bio- and serotypes of *Y. pseudotuberculosis* are pathogenic. In Finland, *Y. pseudotuberculosis* used to be rare causing only sporadic infections. However, since 1997 it has caused five outbreaks infecting almost 300 individuals (Hallanvuo *et al.* 2002).

2.3. Viruses and parasites

Many of the infections caused by viruses and parasites are undetected. Diagnostic tests for these pathogens are not available in routine laboratories in Finland and many other countries. However, it has been estimated that viral pathogens account for 80% of enteric illnesses caused by known pathogens in the United States (Mead *et al.* 1999). The main viruses that cause food-borne illnesses are norovirus (previously also called Norwalk-like virus or calicivirus), rotavirus and astrovirus (Mead *et al.* 1999; Adak *et al.* 2002). In Finland, the most common virus in sporadic infections is rotavirus, around 1,000 to 1,500 cases each year (http://www.ktl.fi/ttr). However, the norovirus has been the most common cause of food-borne outbreaks (Hatakka *et al.* 2002). The proportion of food-borne norovirus outbreaks varies greatly from one country to another due to the differences in case definition, surveillance systems and methods used (Koopmans *et al.* 2002). Many outbreaks in Finland and elsewhere have been connected to drinking water (Kukkula *et al.* 1999; Boccia *et al.* 2002; Anderson, A. D. *et al.* 2003). Contaminated imported frozen berries have also been common vehicles in Finland (Kukkula 1998; Hatakka and Wihlman 1999; Pönkä *et al.* 1999; Hatakka and Halonen 2000; Hatakka *et al.* 2001; Hatakka *et al.* 2002).

The food-borne parasites include *Protozoans* and helminths. In the United States, it has been estimated that 7% of enteric illnesses caused by known pathogens are due to parasites and the most common parasite has been *Giardia lamblia* (Mead *et al.* 1999). The parasitic sporadic infections and outbreaks are often associated with child day care centres and consumption of untreated surface water (Steketee *et al.* 1989; Dennis *et al.* 1993). *G. lamblia* is also the most common parasite in Finland causing around 250-300 cases annually (http://www.ktl.fi/ttr). The other parasites recorded in the Finnish National Infectious Disease Register (NIDR) are *Entamoeba histolytica* and *Cryptosporidium*.

The culture of protozoan parasites is highly complex and, for example, enrichment media are not available for cultivating parasites (Ortega 2001; Visvesvara and Garcia 2002). However, other techniques like PCR, RFLP, and techniques to detect antigens, have been developed to improve detection and identification (Ortega 2001).

3. Characterization of bacteria for epidemiological surveillance

The main purpose in epidemiology is to differentiate documented outbreaks and sporadic cases and, furthermore, detect outbreaks in a population. Prevention and control of food-borne diseases very much depend on early outbreak recognition that good surveillance systems can provide. The research carried out in microbiology laboratories is an important part of food-borne disease surveillance (Cowden 1996) and is constantly and significantly increasing. In outbreak situations, the epidemiologists can, for example, use the microbiological subtyping results to identify linked cases, make plans and outline the case-control studies. Moreover, the cooperation in harmonisation of typing methods at the intrernational level has increased (McLauchlin *et al.* 1996; Peters *et al.* 2003)

Many of the food-borne bacteria are widely distributed in the environment. Some of them can also be part of the normal flora of human or animals. All these can make the specific diagnostic difficult in epidemiological surveillance. Some bacteria also have the ability or mechanisims, like sporulation, to survive for long periods under adverse environmental conditions including cooked food. The bacteria have many metabolic or biological activities that can be used in characterization. Furthermore, virulence mechanisms, which give bacteria the ability to cause infections, can also be investigated by studying genetic elements of bacteria. To better understand the origin and spread of bacterial diseases, it is also important to define the relationships that exist between virulent and their non-virulent counterparts of strains (Joyce *et al.* 2002).

Detailed strain identification is essential for successful epidemiological studies of the surveillance of sporadic gastrointestinal illnesses and food-borne outbreaks. Traditionally, researchers have relied on phenotypic methods. However, these classical methods are not always discriminatory enough for epidemiological purposes. Modern typing methods are based on the characterization of the genotype of the organism.

The optimal typing method in a laboratory will depend on several factors. Ideally, the typing method should have high typeability, reproducibility and discriminatory power. It should be simple to perform as should the interpretation of results. The cost must also be taken into account. van Belkum *et al.* (2001) have listed, from a theoretical perspective, the qualites of microbial typing techniques mentioned above (Table 7).

3.1. Phenotyping methods

Phenotypic methods detect the presence or absence of metabolic or biological activities as expressed by microorganisms. These methods include, for example, biotyping, serotyping, phage typing, antimicrobial sensitivity testing, immunoblotting and MLEE (multilocus enzyme electrophoresis). Many phenotyping methods are widely available, easily performed and relatively inexpensive. However, all available specific reagents are not necessarily suitable for all strains. Therefore, many strains might be untypeable. Moreover, the discriminatory power is often not sufficient for epidemiological studies.

3.1.1. Biotyping

Biotyping includes metabolic activities expressed by an isolate and may include specific biochemical reactions, colonial morphology and environmental tolerances. Biotyping has only a limited ability to differentiate among strains within species

Table 7. Characteristics of microbial typing methods from a theoretical perspective. Adapted from van Belkum *et al.* (2001) with modifications.

Typing method[a]	Typeability	Reproducibility	Discriminatory power	Ease of performance	Ease of interpretation	General availability	Cost
Phenotypic							
Antimicrobial susceptibility	Good	Good	Poor	Excellent	Excellent	Excellent	Low
Manual biotyping	Good	Poor	Poor	Excellent	Excellent	Excellent	Low
Automated biotyping	Good	Good	Poor	Good	Good	Variable	Medium
Serotyping	Variable	Good	Variable	Good	Good	Variable	Medium
PAGE	Excellent	Good	Good	Excellent	Fair	Good	Medium
MLEE	Excellent	Excellent	Good	Good	Excellent	Variable	High
Bacteriophage typing	Variable	Fair	Variable	Poor	Poor	Excellent	Medium
Genotypic							
Plasmid profiles	Variable	Fair	Variable	Fair	Good	Excellent	Medium
Plasmid REA	Variable	Excellent	Good	Good	Excellent	Excellent	Medium
Chromosomal REA	Excellent	Variable	Variable	Good	Fair	Variable	Medium
Ribotyping	Excellent	Excellent	Good	Good	Good	Variable	High
PFGE	Excellent	Excellent	Excellent	Good	Good	Variable	High
PCR	Excellent	Fair	Excellent	Good	Fair	Good	Medium
AFLP	Excellent	Good	Excellent	Good	Fair	Low	High
DNA sequencing	Optimal	Excellent	Excellent	Poor	Excellent	Low	High

[a] PAGE, polyacrylamide gel electrophoresis; MLEE, multilocus enzyme electrophoresis; REA, restriction endonuclease analysis; PFGE, pulsed-field gel electrophoresis; PCR, polymerase chain reaction; AFLP amplification fragment length polymorphisim

and has relatively poor discrimination power in epidemiological studies. However, biotyping especially supports the results of serotyping in the characterization of the pathogenic strains of *Y. enterocolitica* (Bottone 1999).

3.1.2. Serotyping

Serological typing is based on different variations of antigenic determinants expressed on the cell surface, including lipopolysaccharides (LPS), capsular polysaccharides, membrane proteins and extracellular organelles (flagella and fimbriae). Somatic antigens have been marked with O, flagellar with H and capsular with Vi. This typing method has been applied to both gram-negative, e.g. *Salmonella* and *E. coli,* and gram-positive, e.g. *Listeria*, organisms.

Serotyping is a classical tool for epidemiological studies (Filice *et al.* 1978). However, in a modern survey of microbes it should be used together with more discriminatory methods since its value alone is limited. Although the epidemiological benefit is limited, it has provided rapid information for the screening of isolates during suspected outbreaks (Lyytikäinen *et al.* 2000a). The preparation of specific antisera is a difficult process, if commercial reagents are not available. Therefore, in such cases usually only reference laboratories have prepared specific antisera.

The present knowledge on the antigenic structure for serological differentiation of *L. monocytogenes* was represented by Seeliger and Höhne (1979). Serotyping identifies 13 serotypes of *L. monocytogenes*. Serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c of *L. monocytogenes* need identification of both O and H antigens, whereas serotypes 4a, 4ab, 4c, 4d, 4e and 7 have the same H antigens and, therefore, only need identification of O antigens (Table 8).

Table 8. Serotypes of *L. monocytogenes* and their O:H antigens (Seeliger and Höhne 1979)

Serotype	O-Antigens	H-Antigens
1/2 a	I, II, (III)	AB
1/2 b	I, II, (III)	ABC
1/2 c	I, II, (III)	BD
3 a	II, (III), IV	AB
3 b	II, (III), IV, (XII), (XIII)	ABC
3 c	II, (III), IV, (XII), (XIII)	BD
4 a	(III), (V), VII, IX	ABC
4 ab	(III), V, VI, VII, IX, X	ABC
4 b	(III), V, VI	ABC
4 c	(III), V, VII	ABC
4 d	(III), (V), VI, VIII	ABC
4 e	(III), V, VI, (VIII), (IX)	ABC
7	(III), XII, XIII	ABC

Salmonella isolates can be divided into approximately 2,500 serotypes by serological differentiation (Popoff 2001). The antigenic structures are listed in the Kauffman-White scheme and are expressed as follows: O antigen(s), Vi (when present): H antigen(s) (phase 1): H antigen(s) (phase 2, when present).

3.1.3. Phage typing

Phage typing is based on the capability of a standard set of viruses to infect and lyse bacterial cells. Phage typing is usually available only in reference laboratories. It is a rapid and economical method (Ward *et al.* 1987). The phage typing scheme has been established, for example, for *S.* Enteritidis (Ward *et al.* 1987), *S.* Typhimurium (Anderson, E. S. *et al.* 1977b) and *S.* Virchow (Chambers *et al.* 1987).

In the WHO study on subtyping isolates of *L. monocytogenes* by phage typing, limited comparability between results was obtained from testing the same cultures using the same phages in six different laboratories (McLauchlin *et al.* 1996). It was concluded that better inter-laboratory reproducibility might be achieved by the standardization of phage suspensions, propagation strains and methodology, together with the use of centrally propagated phages. However, patterns of phage susceptibility were relatively stable on retesting strains in the same laboratory after long periods of time (McLauchlin *et al.* 1996).

3.1.4. Determination of antimicrobial resistance patterns

Clinical microbiology laboratories routinely test most isolates for their susceptibility to different antibiotics. The discriminatory power of antibiotic susceptibility tests is poor, but it is often the first indicator of an outbreak if the pattern of antibiotic resistance is new or unusual and has been detected in the isolates of many patients. Moreover, the use of antibiotic susceptibility testing in subtyping is limited because strains under selective pressure can get (or lose) specific resistance genes via plasmids and transposons from other strains or species (Brown *et al.* 1991). For example, strains of *S.* Typhimurium (Anderson, E. S. *et al.* 1977a) and *S.* Johannesburg (Chau *et al.* 1982) have been reported to lose their antimicrobial drug resistance during transfer or storage. Problems also appear when the *in vitro* susceptibility of isolates does not correlate with the situation *in vivo*.

3.1.5. Detection of toxin production

Various enzyme immune assays can be used for the analysis of bacterial toxins (Feng 2001). Recently, the reversed passive latex agglutination (RPLA) assay has become commercially available for such as *B.cereus*, *C. perfringens* and EHEC. In the reversed agglutination assay, the antibody attached to the latex particles reacts with the soluble antigen.

The expression of enterotoxin of *C. perfringens* is connected to sporulation. However, sporulation is difficult to achieve when isolates grow in laboratory media. Therefore, the detection of the enterotoxin of *C. perfringens* has not been performed routinely in many laboratories (Harmon and Kautter 1986; Katayama *et al.* 1996).

3.2. Genotyping methods

Genotyping methods account for DNA-based analyses of chromosomal or extra chromosomal genetic elements. Therefore, the possible genetic changes, such as point mutations, insertions, deletions and plasmid transfer must be taken into account when typing results are interpreted. Mutations are belived to be random with respect to their phenotypic effect. However, in recent years microbial genomes are believed to have far greater mutational flexibility than was previously assumed (van Belkum *et al.* 2001).

Genotypic methods include, for example, plasmid analysis, ribotyping, polymerase chain reaction (PCR), Pulsed-field gel electrophoresis (PFGE) and amplification fragment length polymorphisim (AFLP).

The selection of the suitable and most applicable typing technique depends on the purpose of the analysis. PFGE is widely used especially in epidemiological studies to characterise genetically closely related strains. Although PFGE analyses the whole genome, it provides only limited information of the genetic characteristics of the strain. Ribotyping utilizes the similarities and differences found in the ribosomal RNA genes and their surrounding sequences. These genes are highly conserved and are called "housekeeping genes" which are important for cell division and reproduction. Ribosomal RNA genes are present in all bacteria and have become the best targets for studing phylogenetic relationships (Ludwig and Schleifer 1994; Vandamme *et al.* 1996). PCR can be used when a selected region of a genome is studied. However, the target to be amplified should be chosen with care and should be connected only to the pathogen studied. The full-genome sequences have opened new opportunities to study bacteria and diseases. So far, the whole genome of more than 1,000 viruses and 100 other microbes have been partly or completely sequenced (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Sequencing allows us to catalogue all genetic variables giving us knowledge of bacterial pathogenity and helps us to understand better the origin and spread of microbial diseases. Based on sequencing of specific housekeeping genes, a method called MLST (multi locus sequrence typing) has been developed and used for strain typing with the aid of sequence databases for this specific purpose. Another new technique that makes use of large-scale genome sequencing is DNA microarrays. An array is a collection of sequences representing specific genomes of interest. Arrays that discriminate closely related strains and recognize genetic markers can be constructed and help to understand the epidemiology and possible pathogenesis of the strains. In this review of literature we focus on methods used in this dissertation.

3.2.1. Plasmid analysis

Plasmid analysis can include typing of plasmid profile, plasmid fingerprinting and identification of plasmid-mediated virulence genes. Plasmid profiling is based on the number and characteristic molecular weight of plasmids (Liebana *et al.* 2001b; Horby *et al.* 2003). In plasmid finger-printing, the plasmid DNA is digested with restriction enzymes (Tassios *et al.* 1997). Many pathogens have plasmids associated with virulence and are characteristic of these pathogens. The determination of molecular weights of these plasmids can be useful markers (Horby *et al.* 2003). For example, *Salmonella* and *Shigella* carry certain "serotype-specific" plasmids: *S.* Enteritidis carries a plasmid of 38 MDa (Chart *et al.* 1989b), *S.* Typhimurium a 60 MDa plasmid (Helmuth *et al.* 1985), *S. sonnei* has a 120 MDa plasmid (Sansonetti *et al.* 1981) and *S. flexneri* a 140 MDa plasmid (Sasakawa *et al.* 1986).

The disadvantage of plasmid analysis is that many pathogens can spontaneously lose or acquire plasmids (Brown *et al.* 1991). This limits the use of plasmid analysis for epidemiological investigations.

3.2.2. Polymerase chain reaction (PCR)

Polymerace Chain Reaction (PCR) was described by Saiki *et al.* (1985) and Mullis and Faloona (1987). PCR is an *in vitro* method for enzymatically synthesizing DNA from a selected region of a genome to be amplified. This particular DNA sequence is called template and at least part of its nucleotide sequence is already known. The template should represent a relatively small fragment of DNA, typically 0.2 to 2.0 kb, because larger target sequences are difficult to amplify efficiently. The PCR technique can multiply DNA molecules rapidly and exponentially up to one billion-fold, yielding large amounts of specific DNA sequences. The known part of the sequence is used to design two synthetic DNA oligonucleotides, primers, one complementary to each strand of the DNA double helix. These primers determine the ends of the final DNA fragment when they bind to complementary DNA strands and they are catalysed by a DNA polymerase to extend these complementary DNA strands. A cycle of replication consists of three thermal steps; denaturing of the double-stranded DNA template, binding of primers to each strand of the template, and synthesizing of the complementary strand. This new DNA sequence will be the template in the next cycle. According to Xu and Larzul (1991), in theory DNA will be amplified 2^n when n is the number of cycles.

The concentration and design of the sequences of primers are the most important steps in the success of the overall PCR assay. The other crucial thing is the melting temperature of the primers. Therefore, several primer design software programs are available.

One advantage in PCR is that it can be used to detect and identify non-culturable microorganisms (Fredricks and Relman 1999). It is also a very sensitive method and therefore, although the sample concentration is low, the target genes can be detected. However, because of the sensitivity of PCR it is also sensitive to the contamination of foreign DNA and can, therefore, give false positive results (Fredricks and Relman 1999).

Many different applications of PCR have been described and PCR can offer several advantages over other nucleic acid-based typing methods. Arbitrarily primed PCR (AP-PCR) or random amplified polymorphic (RAPD) are methods that use PCR for creating genomic fingerprints by the amplification of randomly chosen sequences. Repetitive sequences occurring in many bacterial genomes have also been used to design primers suitable for genomic fingerprinting of bacteria (rep-PCR). In restriction fragment length polymorphism (PCR-RFLP), a known sequence is amplified, cut with restriction entzyme and the restriction fragments are compared between different strains. Amplified fragment length polymorphism (AFLP) is the most recently adopted PCR-based typing technique. In this method the genomic DNA is digested with two enzymes, a frequently cutting and a less frequently cutting enzyme. Then the resulting fragments are ligated to specific adaptors and amplified using primers recognizing the adapters. Finally, the labelled products are separated by electrophoresis. In addition, with PCR it is simple to accumulate a large amount of material that can be sequenced directly after purification.

3.2.3. Ribotyping

Ribotyping utilizes the similarities and differences found in the ribosomal genes and their surrounding sequences. These genes are highly conserved and vary in number and position within the genome of bacteria. The chromosomal DNA is isolated and digested with a frequently cutting enzyme. Then the DNA fragments, carrying rRNA genes, is separated from other fragments by electrophoresis on agarose gels and detected by hybridization with labelled *Escherichia coli* 16S, 23S or 5S probes.

An automated ribotyping system has been introduced (Bruce 1996). This automated application has several benefits over manual ribotyping including increased standardization, lower labour costs, increased speed, better between-run comparison and decreased number of non-typeable isolates (Hollis *et al.* 1999; Suihko *et al.* 2002). Automated ribotyping has been used to characterize isolates of e.g. *L. monocytogenes* (Allerberger and Fritschel 1999), *S.* Enteritidis (Clark *et al.* 2003), *S.* Typhimurium (De Cesare *et al.* 2001b) and *C. jejuni* (de Boer *et al.* 2000).

3.2.4. Pulsed-field gel electrophoresis (PFGE)

The PFGE (pulsed-field gel electrophoresis) method was developed by Schwartz and Cantor (Schwartz, D. C. and Cantor 1984). This method is a variation of conventional agarose gel electrophoresis where the direction of the electric field current is constant. In PFGE, the electrical field switches the direction of the current according to a predetermined program and timetable. Thus, the fragments during the migration take time both for reorientation and movement in the electric field. The reorientation takes more time for a large fragments, thus, allowing even the separation of 12 Mb (Farber 1996) DNA fragments. The migration of DNA fragments can be affected by varying the concentration of the agarose gel, the switch of pulse times, the voltage gradients, the angle of the electric field, the running time and temperature (Black *et al.* 1988; Birren and Lai 1993).

In PFGE, the bacteria are embedded in agarose and lysed *in situ',* which protects the chromosome from mechanical break damage. The chromosomal DNA is digested with a restriction endonuclease, which recognizes and cuts infrequently 6- or 8-base sequences. These enzymes cleave the chromosomal DNA into between 5 to 30 fragments. For *Salmonella*, the restriction enzyme *Xba*I (Lyytikäinen *et al.* 2000b; Liebana *et al.* 2001a) has been the most used enzyme in PFGE, for *C. perfringens* the enzymes *Spe*I (Ridell *et al.* 1998; Maslanka *et al.* 1999) and *Apa*I (Collie and McClane 1998; Ridell *et al.* 1998) and for *L. monocytogenes* the enzyme *Asc*I (Brosch *et al.* 1996; Graves and Swaminathan 2001).

PFGE has attained great value in epidemiological analysis, the differentiation of pathogenic strains and in monitoring their spread among communities (Gautom 1997). Currently, PFGE seems to be the most widely used subtyping method and is often the standard one to which other methods are compared. PFGE is a very accurate and reproducible method. However, in PFGE the whole bacterial genome is digested by the infrequently cutting enzyme and the profiles will differ if one base changes at the recognizition site. Problems can thus caused by recent point mutation, deletion, insertion and loss or acquisition of plasmids within a subtype, which all might account for minor differences. These changes usually result in two to three fragment differences in banding patterns. Therefore, one of the generally accepted interpretation rules is that an isolate is

closely related to an outbreak strain when the fragment difference is around two to three fragments, possible related when it is four to six and unrelated when the difference is seven or more (Tenover *et al.* 1995). DNA degradation is also a problem in PFGE for some strains, as in conventional electrophoresis (Ray *et al.* 1992; Kristjansson *et al.* 1994; Ridell *et al.* 1998; Marshall *et al.* 1999; Corkill *et al.* 2000; Römling and Tummler 2000; Fawley and Wilcox 2002; Klaassen *et al.* 2002; Liesegang and Tschäpe 2002). One of the other disadvantages of this method is the time needed to finish the assay and its labour intensive. However, a recently described standardized protocol (Gautom 1997) made it possible to complete the subtyping process within 24 to 30 hours. Therefore, PFGE is still one of the best methods available for surveillance purposes having high discriminatory power with reproducible results.

PFGE has become a standard technique among public health laboratories. To increase further the epidemiological surveillance at the national and international level, the PFGE method for different pathogens has been harmonized, for example, the Harmony-project for *S. aureus*; (van Belkum *et al.* 1998), the CampyNet-project (www.svs.dk/campynet/) for *Campylobacter* and the Salm-gene-project for *Salmonella* (Peters *et al.* 2003). Electronic databases of PFGE profiles have also been created. PulseNet in the USA has already demonstrated the value of an electronic network of PFGE profiles of human foodborne pathogens (EHEC O157:H7, non-typhoidal *Salmonella* serotypes, *L. monocytogenes* and *Shigella*), for early recognition of outbreaks and rapid identification of their sources (Swaminathan *et al.* 2001).

AIMS OF THE STUDY

The general aim of this work was to design and compare a set of methods to gain detailed information of the diversity of the food-borne bacteria important for the public health. The specific aims were:

1. To develop the optimal PFGE protocol for genotyping of the species and groups in focus, by for example, finding conditions to prevent the degradation of DNA, a major problem in PFGE genotyping.

2. To genotype outbreak- and nonoutbreak-associated strains of *S. enterica* serotype Enteritidis phage types 1 and 4 in order to investigate potential homogeneity or heterogeneity between isolates within each phage type.

3. To set up i) the diagnostic methods that can be used to separate the *C. perfringens* food poisoning isolates from the isolates of human normal flora and ii) the PFGE genotyping method to give further information about the strains for epidemiological purposes.

4. To evaluate the usefulness of the combination of two genotyping methods (PFGE and ribotyping) for subtyping *L. monocytogenes* strains.

5. To study the distribution of pheno-and genotypes of *L. monocytogenes* among strains of human and non-human origin.

6. To construct a database library of the PFGE profiles of *L. monocytogenes* strains in order to improve the detection of the potential infection clusters caused by this bacterium.

MATERIALS AND METHODS

1. Bacterial strains (I-V)

The study included 551 strains belonging to four species within four genera (Table 9). In studies I-V, strains had been stored at -70ºC in sterilized skim milk. In study I, some strains had been stored also at room temperature in nutrient agar tubes and in study III, industrial isolates in 5% glycerol. In all the studies, one isolate per sample was studied.

Study I. *S.* Enteritidis PT1 domestic human strains included six strains related to the outbreaks in two Finnish municipalities (Vieremä and Turku) and two animal isolates related to an outbreak in a Finnish commercial layer flock (Johansson *et al.* 1996). Other animal isolates (two) were from Finnish cows. For comparison, isolates associated with travelling abroad were also included. Eleven strains were from Eastern Europe; four of them were obtained from Baltic countries (kindly sent by Dr. Unna Jöks, Central Laboratory of Microbiology, Health Protection Inspectorate, Tallinn, Estonia) and 13 strains from Western Europe. *S.* Enteritidis PT4 isolates included six strains related to the outbreaks in three Finnish municipalities (Leppävirta, Kotka and Rauma). Sixteen human strains were associated with travelling in Europe and two strains outside Europe. Eight strains were domestic animal isolates (six from cows and two from turkeys) and three strains were isolated from imported animals (pig, chicken and turkey). In addition, two reference strains were used as controls in the plasmid analysis (Table 10).

Study II. All *C. perfringens* isolates were related to nine food-borne outbreaks that occurred from 1984 to 1999. Additionally, three reference strains were used as controls in PCR (Table 10).

Studies III and IV. All 314 *L. monocytogenes* human isolates were associated with infections diagnosed from 1990 to 2001 in patients living throughout Finland. For comparison, 72 food industrial isolates were collected from 11 food-processing plants from 1997 to 1999.

Study V. Four of the nine *S.* Ohio strains represented an outbreak (three human and one environmental isolates) and five strains were sporadic human isolates. All seven *S.* Newport strains were connected to an outbreak (Lyytikäinen *et al.* 2000b). Two *E. coli* strains were associated with infections in patients in 1999.

Table 9. Bacterial strains. Total number in each group is written in bold.

Strain	Origin		No. of isolates (n = 551)	Study
Salmonella Enteritidis PT1			**57**	I
	Human	Domestic	26	
		Foreign	25	
	Animal	Domestic	2	
		Foreign	3	
	Foodstuffs	Foreign	1	
Salmonella Enteritidis PT4			**43**	I
	Human	Domestic	14	
		Foreign	18	
	Animal	Domestic	8	
		Foreign	3	
Salmonella Ohio			**9**	V
	Human	Domestic	4	
		Foreign	4	
	Environmental	Domestic	1	
Salmonella Newport			**7**	V
	Human	Domestic	7	
Escherichia coli			**2**	V
	Human	Domestic	2	
Clostridium perfringens			**47**	II
	Human	Domestic	40	
	Foodstuffs	Domestic	7	
Listeria monocytogenes			**386**	III-IV
	Human	Domestic	314	
	Food industry	Domestic	72	

Table 10. Reference strains used as controls.

Strain	Relevant property	Reference	Study
Escherichia coli			
RH 4240	Plasmid-containing strain (39R861)	Threlfall *et al.* 1986	I
RH 4241	Plasmid-containing strain (V517)	Macrina *et al.* 1978	I
Clostridium perfringens type A			
RH 4819	Positive for *cpe*	NCTC 8238	II
RH 4820	Positive for *cpe*	NCTC 8239	II
RH 4847	Negative for *cpe*	ATCC 3624	II

2. Primers, restriction enzymes and molecular weight standards (I-V)

The sequences of the oligonucleotide primers for the *cpe* enterotoxin gene were selected (Buogo *et al.* 1995) from the sequence originally published by Van Damme-Jongsten *et al.* (1989), taking into account the differences in the publication of Czeczulin *et al.* (1993). The primers were as follows: 5'-TAA CAA TTT AAA TCC AAT GG-3' and 5'-ATT GAA TAA GGG TAA TTT CC-3'. The size of an amplified fragment was 933 bp. Seven restriction enzymes were used in PFGE and one restriction enzyme in automated ribotyping (Table 11).

Table 11. Restriction enzymes.

Method	Enzyme	Manufacturer	Specificity 5' → 3'	Study
PFGE				
	*Apa*I	MBI Fermentas Ltd., Vilnus, Lithuania	GGGCC C	II
	*Asc*I	New England BioLabs Inc., Beverly, Mass. USA	GG CGCGCC	III, IV
	*Bln*I	Boehringer Mannheim GmbH, Mannheim, Germany	C CTAGG	V
	*Not*I	Boehringer Mannheim GmbH, Mannheim, Germany	GC GGCCGC	I
	*Sma*I	Boehringer Mannheim GmbH, Mannheim, Germany	CCC GGG	II
	*Spe*I	Boehringer Mannheim GmbH, Mannheim, Germany	A CTAGT	I
	*Xba*I	Boehringer Mannheim GmbH, Mannheim, Germany	T CTAGA	I, V
Ribotyping				
	*Eco*RI	DuPont Qualicon, Wilmington, DE, USA	G AATTC	III

Table 12. Molecular weight standards.

Marker	Manufacturer	Size range (kb)	Study
Lambda ladder	New England BioLabs Inc., Beverly, Mass. USA	50 - 1000	I, II, V
Low Range PFG Marker	New England BioLabs Inc., Beverly, Mass. USA	0.1 - 200	II, III, IV
Molecular weight marker[a]	DuPont Qualicon, Wilmington, DE, USA	0.9 - 48	III
pUC Mix Marker 8	MBI Fermentas Ltd., Vilnus, Lithuania	0.02 - 1.1	II

[a] Molecular weight marker is included in commercial DNA Prep pack kit.

3. Antimicrobial susceptibility testing (I)

The antimicrobial susceptibility of the *S.* Enteritidis PT1 and PT4 strains was determined by the agar diffusion method on a semisynthetic Iso-Sensitest medium to the following 12 antimicrobial agents (Casals and Pringler 1991): ampicillin, chloramphenicol, ceftriaxone, imipenem, mecillinam, nalidixic acid, neomycin, sulfonamide, tetracycline, trimethoprim, streptomycin and ciprofloxacin (Neo-Sensitabs, AS Rosco, Taastrup, Denmark). The interpretation of inhibition zones was according to the recommendations of the Swedish Reference Group for antibiotics (Anonymous 1990).

4. Serotyping (III, IV)

Strains of *L. monocytogenes* were serotyped using antisera against O and H antigens according to the instructions of the manufacturer (Denka Seiken Co., Ltd, Tokyo, Japan) with minor modifications. The strains were taken from –70°C and revived on sheep blood agar (Oxoid, Hampshire, England) overnight at 37°C before inoculation on brain heart infusion (BHI) agar (Difco, Detroit, MI) for the determination of the O antigens. The bacterial suspension in 0.2% NaCl was heated at 100ºC for 1 hour, instead of 121ºC for 30 minutes. For the determination of the H antigens, the strains were passed at 25ºC through semi-liquid BHI medium in Craigie's tubes (0.2% agar) four times, instead of three times.

5. Reversed passive latex agglutination (RPLA) (II)

The CPE enterotoxin produced by type A *C. perfringens* strains was detected by reversed passive latex agglutination according to the instructions of the manufacturer (PET-RPLA kit, Oxoid Ltd., Hampshire, England). Modified Duncan and Strong medium (1968) was used for sporulation. If the production of entero-toxin was not detected or production was weak when the result of PCR was positive, the growth of the strains were checked by culturing one loopful (10µl) of Cooked Meat medium overnight anaerobically on Brucella sheep-blood agar plate at 37°C, before and after heat treatment at 75°C for 20 minutes. The sporulation of these strains after culturing in modified Duncan and Strong medium was examined under the microscope.

6. Plasmid profiling (I)

The plasmid DNA of *S.* Enteritidis PT1 and PT4 strains was isolated by the alkaline lysis method as described by Grinsted and Bennet (1988). Samples were analysed by electrophoresis at 120 V for 1 h 10 min on 0.9% horizontal Sea Kem ME agarose gels (FMC BioProducts). Plasmid-containing strains RH 4240 (39R861, Threlfall *et al.* 1986) and RH 4241 (V517, Macrina *et al.* 1978) were used as controls (Table 10). Plasmid profiles were given a lower case letter starting with a.

7. PCR (II)

The primers for the *cpe* gene and the PCR method described by Buogo *et al.* (1995) were used. Briefly: the template DNA for the amplification was obtained from the *C. perfringens* bacteria using a direct lysis method, and two µl of lysed cells was added to PCR mix. PCR was performed in Eppendorf Mastercycler Gradient (Hamburg, Germany) or Hybaid PCR Sprint Temperature Cycling (Ashford, Middlesex, UK). The following procedure was used: initial denaturation at 95°C for 10 min, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 46°C, and 30 s at 72°C each. The final step was a 10-min incubation at 72°C. The amplification products were analysed by electrophoresis at 90V for 1h and 15 min in a 2% SeaKem ME agarose gel (FMC BioProducts, Rockland, ME) using a GIBCO BRL Horizon 20.25 system (Life Technologies Inc., Gathersburg, MD). The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination. pUC Mix Marker 8 (MBI Fermentas Ltd., Vilnius, Lithuania) was used as a molecular weight standard (Table 12). The size of an amplified fragment was 933 bp.

8. Isolation of chromosomal DNA in PFGE (I - V)

In study I, *Salmonella* strains were grown overnight on Drigalski-Conradi agar plates at 37°C. Bacterial cells were suspended in 1200 µl of TEN buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.1 M EDTA [pH 7.5]) to an optical density at 600 nm of 0.150-0.200. In study II, *C. perfringens* strains were grown anaerobically on egg yolk agar (EYA) plates overnight at 37°C and then for 5 h at 37°C in trypticase glucose yeast extract (TGY) broth. In studies III and IV, *L. monocytogenes* strains were grown on sheep blood agar overnight at 37°C and then for 17-18h at 37°C in BHI broth. In studies II, III and IV, 2 ml of broth culture was mixed with 5 ml of cold PIV buffer (10 mM Tris [pH 7.5], 1 M NaCl). The mix was centrifuged for 15 min at 4˚C 3000 rpm and the pellet was suspended with 750 µl of cold PIV buffer. Cell suspension in studies I, II, III and IV was mixed in equal parts with molten 2% low-melting-point agarose (Sea Plaque agarose FMC BioProducts, Rockland, Maine USA) and the mixture was pipetted into plug moulds. In study I, the plugs were incubated overnight at +55°C - +57°C in ES buffer (0.5 M EDTA, 1% N-lauroylsarcosine) with 0.15 mg of proteinase K per ml. In studies II, III and IV, the plugs were incubated overnight at 37°C in EC buffer (6mM Tris-HCl [pH 7.5], 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine) with 1 mg of lysozyme per ml and again overnight at 55 to 57°C in ES buffer with 0.3 mg of proteinase K per ml. The plugs were first washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) for 30 min, then with TE buffer and 4 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min to inactivate the proteinase K, and then again three times with TE buffer for 30 min.

In study IV for some of the strains and in study V for all the strains, the shorter protocol for the preparation of genomic DNA described by Gautom (1997) (study V) and Graves and Swaminathan (2001) (study IV) was used with slight modifications. In study IV, in the shorter protocol, the bacterial cells were suspended in 2 ml of CBS (100 mM Tris, 100 mM EDTA [pH 8.0]) to an optical density at 450 nm of 0.7 to 0.8 and in study V, to an optical density at 450 nm of 0.38 to 0.44. When cell suspension (containing lysozyme in study IV) was mixed with low-melting-point agarose, 2% Sea Plaque agarose and Proteinase K was used without 1% sodium dodecyl sulfate.

9. Digestion of chromosomal DNA in PFGE (I - V)

The conditions for restriction endonuclease digestion were essentially those described by Thong *et al.* (1994). In study I, the chromosomal DNA was digested with 10 U of *Xba*I, *Spe*I and *Not*I restriction enzymes (Boehringer Mannheim GmbH, Mannheim, Germany), in study II, with 15 U of *Apa*I (MBI Fermentas Ltd., Vilnus, Lithuania) and *Sma*I (Boehringer Mannheim GmbH, Mannheim, Germany) and in studies III and IV, with 5 U of *Asc*I (New England BioLabs Inc., Beverly, Mass.) (Table 11). All digestions of chromosomal DNA with different enzymes were incubated overnight at 37°C. In study IV, when a shorter protocol was used, incubation time was 4h with 10 U of *Asc*I and in study V, 4h with 10U of *Xba*I and 10U of *Bln*I (Boehringer Mannheim GmbH, Mannheim, Germany) (Table 11).

10. PFGE (I -V)

Electrophoresis was performed with 0.5x TBE (0.045 M Tris-borate 0.001 M EDTA) running buffer in study I at 210V, in study II at 200V and in study V at 178V on 1.0% Sea Kem ME agarose gel (FMC BioProducts, Rockland, Maine, USA), in studies III and IV at 210V on 1.0% Pronadisa D-5 agarose gel (Hispanlab, Madrid, Spain) and in study V also with HEPES (16 mM Hepes-NaOH, 16 mM sodium acetate, 0.8 mM EDTA [pH 7.5]) running buffer at 132V on 1.0% Sea Kem ME agarose gel using the Gene Navigator™ system (Pharmacia, Uppsala, Sweden) or the CHEF Mapper™ systems (BioRad, California, USA). Running conditions in study I for *Xba*I digests were 5 s to 70 s for 24 h, for *Spe*I 5 s to 40 s for 24 h, and for *Not*I 1 s to 20 s for 20 h. In study II, running conditions for *Apa*I and *Sma*I digests were 0.5 s to 40 s for 20 h. In studies III and IV, running conditions for *Asc*I digests were 1s to 28 s for 10 h, followed by 28 s to 30 s for 10h and in study V for *Xba*I and *Bln*I digests 5 s to 70 s for 24 h. In studies I, II and V, Lambda ladder and in studies II, III and IV Low Range PFG Markers (New England BioLabs Inc., Beverly, Mass.) were used as molecular weight standards (Table 12).

11. Ribotyping (III)

The isolates were ribotyped using the automated RiboPrinter® System (DuPont Qualicon™, Wilmington, DE) following the manufacturer's standardized instructions as described by Bruce (1996). Bacterial cells were picked from sheep blood agar (human isolates) or BHI agar (food isolates) plate. DNA was automatically digested by restriction enzyme *Eco*RI (Table 11) and the fragments were hybridised using the *rrnB* rRNA operon from *E. coli* as a chemiluminescent probe (Brosius *et al.* 1981). The automated system processed the batches and generated a pattern for each sample and marker lane using proprietary algorithms. Each batch included six marker lanes including a total of 30 molecular markers which the system

used for the selection of a ribogroup already existing in the database or for the creation of a new one and for the calculation of the similarities between different patterns (software Qualicon version 12.2 (c) 2000). A ribogroup was defined as a set of closely related ribotype patterns (threshold similarity 0.96) that are mathematically indistinguishable from one another by the system (Bruce 1996). The ribogroup patterns represent composite patterns for all members (isolates) of the group analysed with the same instrument. The isolates are called ribotypes and have the same code as the relevant ribogroup. In this work the abbreviations from RT-1 to RT-23 were used. The ribotype patterns of all new ribogroups were analysed three times.

12. Calculation of the discriminatory power (III)

The discriminatory power of a typing method is its ability to distinquish between unrelated strains. The discriminatory power of each typing method was determined by calculating the discriminatory index (DI) by the method of Hunter and Gaston (1988).

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

N is the total number of strains in the sample population, S is the total number of types described, and n_j is the number of strains belonging to the jth type.

13. Electronical clustering and similarity value calculation (III, IV)

Similarity values of PFGE profiles were calculated using the DICE coefficient and clutering was performed according to the unweighted pair-group method using arithmetic averages (UPGMA) available in the BioNumerics software (Applied Maths, Kortrijk, Belgium). UPGMA is the simplest distance method of tree construction and the DICE coefficient measures the similarity based upon common and different bands.

14. Statistical analysis (IV)

Fisher`s exact two-tailed test (Epi-Info 6.04 software; World Health Organization, Geneva, Switzerland and the Centers for Disease Control and Prevention, Atlanta, Ga. USA) was used for statistical analysis. A P value of < 0.05 indicated statistical significance.

RESULTS

1. Preventing DNA degradation in PFGE

Only one of the nine *S.* Ohio strains was typeable by PFGE after restriction of DNA by enzymes *Xba*I and *Bln*I. The other eight strains degraded during the electrophoresis when Tris-containing running buffer TBE was used.

The degradation of DNA was not avoided by modifications of the used standard method using formaldehyde fixation, by increasing the incubation times, by varying the concentrations of proteinase K and by using preincubation with lysozyme.

When another running buffer, HEPES, was used instead of Tris-containing buffer TBE, all *S.* Ohio strains, seven previously untypeable *S.* Newport strains (Lyytikäinen *et al.* 2000b) and two *E. coli* strains were typeable.

The strain, that was typeable with TBE running buffer, shared the same *Xba*I and *Bln*I PFGE type with the strain that was typeable only with HEPES.

When the running voltage was reduced to 5 V/cm in HEPES runs, the gel was comparable to gels run with TBE (6 V/cm) (unpublished).

2. Genotypes and antibiograms of *S.* Enteritidis PT1 and PT4

***S.* Enteritidis PT1.** Seven PFGE profiles (1A-1G) were observed among the 57 PT1 strains studied by PFGE when chromosomal DNAs of the isolates were digested with *Xba*I restriction enzyme (Study I; Fig. 3). All eight outbreak isolates from the outbreaks in Turku and Vieremä belonged to 1F. The most common non-outbreak types were 1A (35%) and 1B (39%), whereas 1C, 1D, 1E and 1G each included only one strain. Seven plasmid profiles (a-g) could be identified by plasmid analysis (Study I; Fig. 4). 82% of the 57 strains, including all eight outbreak strains, had plasmid profile a.

Altogether 12 subtypes (Aa, Ac, Ad, Ba, Be, Bf, Ca, Dg, Eg, Fa, Fb, Gd) were observed among the 57 PT1 isolates when the results of PFGE and plasmid analysis were compiled (Table 13). All outbreak strains belonged to subtype 1Fa. Most of the strains associated with Eastern Europe belonged to subtype 1Aa and those associated with Western Europe to subtype 1Ba. Three strains isolated from domestic cows belonged to subtypes 1Ba (two strains) and 1Eg (one strain) (Table 13). While the plasmid profiles of the subtypes 1Aa, 1Ba and 1Fa were identical, the PFGE profile of 1F differed from 1A and 1B by more than 9 fragments. The fragment difference between PFGE profiles 1A and 1B was four.

Four non-outbreak strains were resistant to some antimicrobials, all the others being sensitive to all antimicrobial agents tested (Study I; Table 1).

***S.* Enteritidis PT4.** Seven PFGE profiles (4A-4G) were observed among the 43 PT4 strains studied by PFGE after *Xba*I restriction of the genomic DNA (Study I; Fig. 5). Of all the strains, including the outbreak strains from Leppävirta, the most common type was 4A (77%). The plasmid analysis identified seven plasmid profiles (a-g) and it distinguished six plasmid profiles within the most common PFGE type 4A (Study I; Fig. 6).

Table 13. *Salmonella* Enteritidis PT1 isolates.

Origin	Geographic origin	No. of Isolates	Subtype[a]	Additional information / source
Human				
	Finland, Turku	4	1Fa	Outbreak isolate
	Finland, Vieremä	2	1Fa	Outbreak isolate
	Finland	8	1Aa	Sporadic isolate
	Finland	7	1Ba	Sporadic isolate
	Finland	1	1Ad	Sporadic isolate
	Finland	1	1Be	Sporadic isolate
	Finland	1	1Ca	Sporadic isolate
	Finland	1	1Dg	Sporadic isolate
	Finland	1	1Fa	Sporadic isolate
	Eastern Europe[b]	7	1Aa	Sporadic isolate[c]
	Latvia	1	1Ad	Sporadic isolate[c]
	Poland	1	1Ba	Sporadic isolate[c]
	Hungary	1	1Fb	Sporadic isolate[c]
	Russia	1	1Gd	Sporadic isolate[c]
	Western Europe[d]	9	1Ba	Sporadic isolate[c]
	Western Europe[e]	2	1Aa	Sporadic isolate[c]
	Germany	1	1Ac	Sporadic isolate[c]
	Germany	1	1Bf	Sporadic isolate[c]
	Spain	1	1Fb	Sporadic isolate[c]
Non-human				
	Finland	1	1Fa	Outbreak isolate, chicken liver[f]
	Finland	1	1Fa	Outbreak isolate, egg shell[f]
	Finland	2	1Ba	Cow, sweep sample
	Finland	1	1Eg	Cow, faeces
	Finland	1	1Ba	Leek

[a] Uppercase letter indicates the PFGE type, and lowercase indicates the plasmid profile
[b] Estonia and other Baltic countries (four strains were provided by the Central Laboratory of Microbiology, Health Protection Inspectorate, Tallinn, Estonia)
[c] From a Finnish tourist who had visited the country in question preceding the finding
[d] Spain, France and Portugal
[e] Norway and Portugal
[f] The strain is from the outbreak in a Finnish commercial layer flock (see reference Johansson *et al.* 1996).

When the results of PFGE and plasmid analysis were compiled, 12 subtypes (Aa, Ab, Ac, Ad, Ae, Ag, Bg, Cb, Db, Eb, Fc, Gf) among 43 PT4 isolates were detected (Table 14). The six outbreak strains were divided into three subtypes: 4Aa (Leppävirta in 1993), 4Bg (Kotka in 1995) and 4Eb (Rauma in 1995) (Table 14). Subtupe 4Aa differed from 4Eb by three PFGE and plasmid fragments and from 4Bg by one PFGE and four plasmid fragments. The difference between subtype 4Eb and 4Bg was four PFGE fragments and one plasmid fragment. The subtype distribution among the non-outbreak strains differed from those above. However, type 4Bg, which was the cause of the outbreak in Kotka in 1995, was isolated from a patient that returned from Spain in 1995; this type was also isolated from a Finnish cow in 1997 (Table 14).

Four non-outbreak strains were resistant to some antibiotics, whereas all other strains were sensitive to all antimicrobial agents tested (Study I; Table 3).

3. CPE toxin production and genotypes of *C. perfringens*

In three suspected infection clusters, IV, VI and VIII, all the strains were CPE- and *cpe*-positive and had an identical PFGE subtype in each cluster (Table 15).

In four suspected infection clusters, II, III, VII and IX, both the outbreak strains and one nonoutbreak strain were detected (Table 15). Furthermore, in suspected infection clusters I and V, all isolates (six and four, respectively) were CPE- and *cpe*-negative and all isolates had different PFGE subtypes (Table 15).

In cluster II, one of the *cpe*-positive strain remained CPE-negative although tested three times for the production of enterotoxin. Also, in cluster VI, one *cpe*-positive strain was tested three times with an uncertain result twice, and the third time only gave a CPE-positive result (Table 15).

All strains carrying the *cpe* gene in the five suspected clusters (III, IV, VI, VIII and IX) had an identical PFGE subtype within each cluster (Cc, Dd, Bb, Aa and Aa, respectively) when digested with *Sma*I and *Apa*I (Table 15). In the suspected cluster II, all three isolates had different PFGE subtypes (Ee, Kk, Ff). In addition, the clusters III, VII and IX included one strain that was *cpe*-negative and belonged to the subtypes Gg, Pp and Qq, respectively (Table 15). In clusters VII, VIII and IX, the same subtype, Aa, caused the outbreaks.

Subtype Aa differed from subtype Dd by three PFGE fragments when digested with *Sma*I (Study II; Fig.1A) and by four when digested with *Apa*I (data not shown). All other subtypes of the *cpe*-positive strains differed from one another by more than 10 fragments. One *cpe*-negative strain, in the suspected clusters III and the other in suspected clusters V, had indistinquishable PFGE profiles (Gg) from each other, whereas all the other *cpe*-negative strains had distinquishable PFGE profiles from each other and from the *cpe*-positive outbreak strains. In clusters II, III, VII and IX, the *cpe*-negative isolates differed by more than 10 fragments from the *cpe*-positive outbreak strain of these clusters when digested with *Sma*I and *Apa*I.

All foodstuff isolates that were connected to the clusters VI and IX belonged to the same subtype Bb and Aa, respectively, as did the human isolates of the same cluster (Table 15).

Table 14. *Salmonella* Enteritidis PT4 isolates.

Origin	Geographic origin	No. of Isolates	Subtype[a]	Additional information / source
Human				
	Finland, Leppävirta	2	4Aa	Outbreak isolate
	Finland, Kotka	2	4Bg	Outbreak isolate
	Finland, Rauma	2	4Eb	Outbreak isolate
	Finland	6	4Ab	Sporadic isolate
	Finland	1	4Ad	Sporadic isolate
	Finland	1	4Fc	Sporadic isolate
	Eastern Europe[b]	3	4Ab	Sporadic isolate[c]
	Hungary	1	4Ae	Sporadic isolate[c]
	Turkey	1	4Db	Sporadic isolate[c]
	Western Europe[d]	6	4Ab	Sporadic isolate[c]
	Europe[e]	2	4Ac	Sporadic isolate[c]
	Germany	1	4Ad	Sporadic isolate[c]
	Spain	1	4Bg	Sporadic isolate[c]
	Italy	1	4Cb	Sporadic isolate[c]
	Outside Europe[f]	2	Ab	Sporadic isolate[c]
Non-human				
	Finland	3	4Ab	Cow
	Finland	2	4Ag	Cow
	Finland	2	4Ag	Turkey
	Finland	1	4Bg	Cow
	Belgium	1	4Ab	Pork, imported from Belgium
	England	1	4Ag	Chicken steak, imported from England
	France	1	4Gf	Turkey, imported from France

[a] Uppercase letter indicates the PFGE type, and lowercase indicates the plasmid profile
[b] Hungary, Lithuania and Turkey
[c] From a Finnish tourist who had visited the country in question preceding the finding
[d] France, Belgium, England, Germany and Spain (Canary Islands)
[e] Cyprus and Spain (Canary Islands)
[f] Thailand and Dominican Republic

Table 15. *Clostridium perfringens* isolates (+; positive property, -; negative property). The strains interpreted to be outbreak-associated are written in bold.

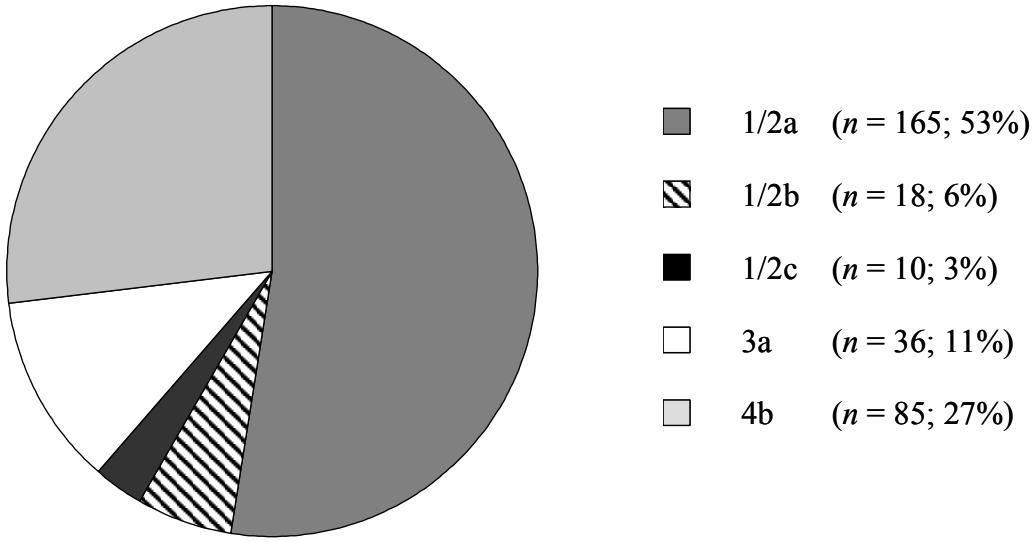
Suspected outbreak	Yr/mo	No. of Isolates	Origin	CPE	Genotype		Interpretation
					cpe type	PFGE type[a]	
I	1984/8	3	Human	-	-	NT	Nonoutbreak strain
I	1984/8	1	Human	-	-	Hh	Nonoutbreak strain
I	1984/8	1	Human	-	-	Ii	Nonoutbreak strain
I	1984/8	1	Human	-	-	Jj	Nonoutbreak strain
II	1988/11	1	Human	+	+	Ee	Outbreak strain
II	1988/11	1	Human	-[b]	+	Ff	Outbreak strain
II	1988/11	1	Human	-	-	Kk	Nonoutbreak strain
III	1992/9	5	Human	+	+	Cc	Outbreak strain
III	1992/9	1	Human	-	-	Gg	Nonoutbreak strain
IV	1993/10	5	Human	+	+	Dd	Outbreak strain
V	1994/8	1	Human	-	-	Ll	Nonoutbreak strain
V	1994/8	1	Human	-	-	Mm	Nonoutbreak strain
V	1994/8	1	Human	-	-	Gg	Nonoutbreak strain
V	1994/8	1	Human	-	-	Nn	Nonoutbreak strain
VI	1997/10	3	Human	+	+	Bb	Outbreak strain
VI	1997/10	2	Foodstuffs[c]	+	+	Bb	Outbreak strain
VII	1998/4	1	Human	+	+	Aa	Outbreak strain
VII	1998/4	1	Human	-	-	Pp	Nonoutbreak strain
VIII	1998/10	8	Human	+	+	Aa	Outbreak strain
IX	1999/3	2	Human	+	+	Aa	Outbreak strain
IX	1999/3	1	Human	-	-	Qq	Nonoutbreak strain
IX	1999/3	5	Foodstuffs[c]	+	+	Aa	Outbreak strain

[a] Uppercase letter indicates the subtype digested with *Sma*I and lowercase letter the subtype digested with *Apa*I. NT; non typeable
[b] Three repetitive determinations for production of enterotoxin gave negative results
[c] Meat casserole

4. Serotypes and PFGE types of human *L. monocytogenes* isolates

The O:H serotyping divided 314 human isolates, collected from 1990 to 2001, into five serotypes: serotype 1/2a (53%), 4b (27%), 3a (11%), 1/2b (6%) and 1/2c (3%) (Figure 6). In 1990, most of the strains were of serotype 4b (61%). Since then, the prevalence of serotype 4b strains has varied between 12 to 44% and strains of serotype 1/2a have been the most common (35-67%) (Study IV; Table 2).

Figure 6. Serotypes of human isolates from 1990 to 2001.



PFGE with restriction enzyme *Asc*I divided 314 strains isolated from humans into 81 PFGE types. The strains of serotypes 1/2a, 1/2b, 1/2c, 3a and 4b were divided into 49, 10, 2, 5 and 18 PFGE types, respectively. Three PFGE types each included one strain belonging to a serotype different from the others. Only 11 of the 81 PFGE types contained five or more strains. When compared with the others, eight of these 11 PFGE types (types 1, 2, 11, 21, 61, 71, 74 and 96) were closely related (difference compared with other types was one to three fragments) to several other PFGE types and were consequently grouped into G1, G2, G11, G21, G61, G71, G74 and G96 (Study IV; Table 3 and Fig. 2). The remaining three PFGE types (5, 24 and 65) were not closely related (difference compared with other types was seven or more fragments) to any other PFGE type and, therefore, each of them formed a group of its own: GT5, GT24 and GT65, respectively. Of the five most common PFGE groups, G1 included seven PFGE types (71 strains; 23%), G71 two (34; 11%), G11 three (32; 10%), G21 eight (29; 9%) and GT5 one (27; 9%) (Study IV; Table 3).

Group G1 (71 strains) formed clusters every year from 1993 onwards (Figure 7). During the period from 1994 to 1996, most of the cases were caused by PFGE types 1 and 23 of group G1 (Study IV; Table 3). In 1997 and 1999, large clusters were formed by PFGE type 1 of group G1 alone. In all, the strains of PFGE type 1 were most common, causing infections every year from 1994 onwards (2 to 11 cases/year).

Group GT5 (27 strains of type 5) formed clusters with at least four cases in 1997, 1998 and 2000 (Figure 7), and four strains in this group GT5 were pregnancy-associated.

Group G71 (34 strains) formed clusters in 1997, 1998 and 1999 (Figure 7). The clusters were formed by serotype 3a, PFGE type 71. Serotype 1/2a, PFGE type 71, caused only one case, which was pregnancy-associated, in 1992.

The 32 pregnancy-associated cases were caused by 17 different PFGE types. PFGE type 24, which belonged to group GT24, was significantly associated with listeriosis in male subjects (9/180 [5%] *vs*. in female subjects 0/132; $P = 0.012$). Otherwise PFGE types were distributed evenly between both genders and among different agegroups.

5. Sero-, ribo-, and PFGE types of human *L. monocytogenes* isolates compared with those of non-human isolates

Seventy-two food industrial isolates collected during a three-year period (1997 to 1999) were analysed and the results were compared with those of all 116 human strains isolated from invasive infections during the same period and representing a subset of the total of 314 *L. monocytogenes* strains isolated in the 1990s. The distribution of O:H serotypes among the 116 subset of the human strains was similar (Figure 8) to that described for all 314 strains (Figure 6). Automated ribotyping with the restriction enzyme *Eco*RI divided 116 human strains into 15 ribotypes. The five commonest ribotypes were RT-2, RT-4, RT-1, RT-5 and RT-19 (Table 16).

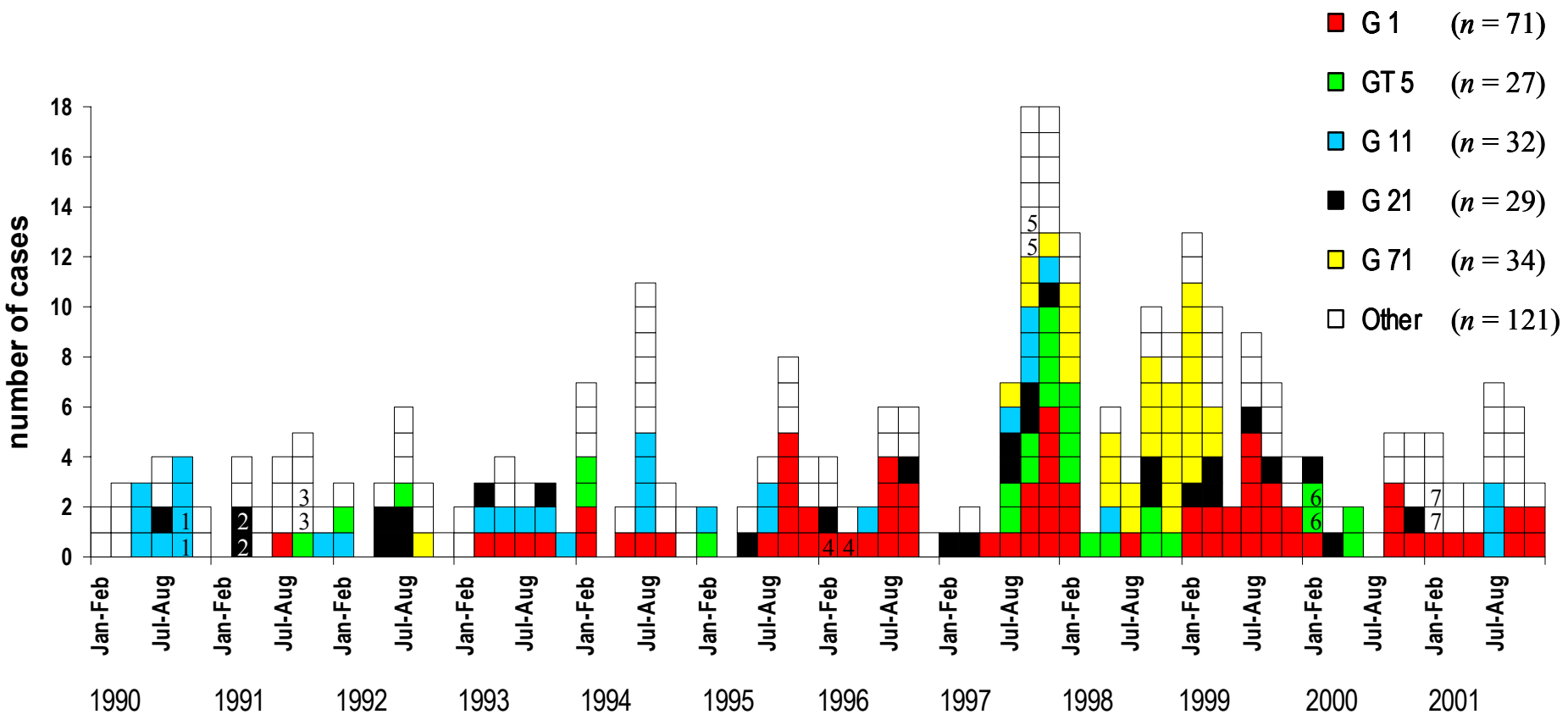
Five O:H serotypes were found among 72 food industrial isolates: 1/2a (79%), 1/2c (7%), 4b (7%). 3a (6%) and 3b (1%). PFGE divided these isolates into 32 PFGE types and automated ribotyping into 15 ribotypes. The five commonest ribotypes were: RT-1, RT-4, RT-6, RT-2 and RT-3 (Table 16).

Table 16. Five commonest types among human ($n = 116$) and food industrial ($n = 72$) isolates collected from 1997 to 1999.

Method (No. of types detected)	Five commonest types (%)
Serotyping	
Human ($n = 4$)	1/2a (51), 3a[a] (25), 4b (23), 1/2b (1)
Non-human ($n = 5$)	1/2a (79), 1/2c (7), 4b (7), 3a (6), 3b (1)
PFGE	
Human ($n = 33$)	71[a] (24), 1 (16), 5 (12), 61 (6), 200 (4)
Non-human ($n = 32$)	2 (7), V21 (7), 96 (7), V4 (7), 1 (6), 200 (6), 42 (6)
Ribotyping	
Human ($n = 15$)	RT-2 (28), RT-4[a] (24), RT-1 (14), RT-5 (7), RT-19 (6)
Non-human ($n = 15$)	RT-1 (19), RT-4 (14), RT-6 (14), RT-2 (13), RT-3 (10)

[a] at least 25 cases were connected to an outbreak from 1998 to 1999 (Lyytikäinen *et al.* 2000a).

Figure 7. Cases of *L. monocytogenes* infections by month from 1990 to 2001. The five most common groups (G1, GT5, G11, G21 and G71) of closely related PFGE types are indicated by colors. The seven mother-child pairs are indicated by numbers (1 to 7).



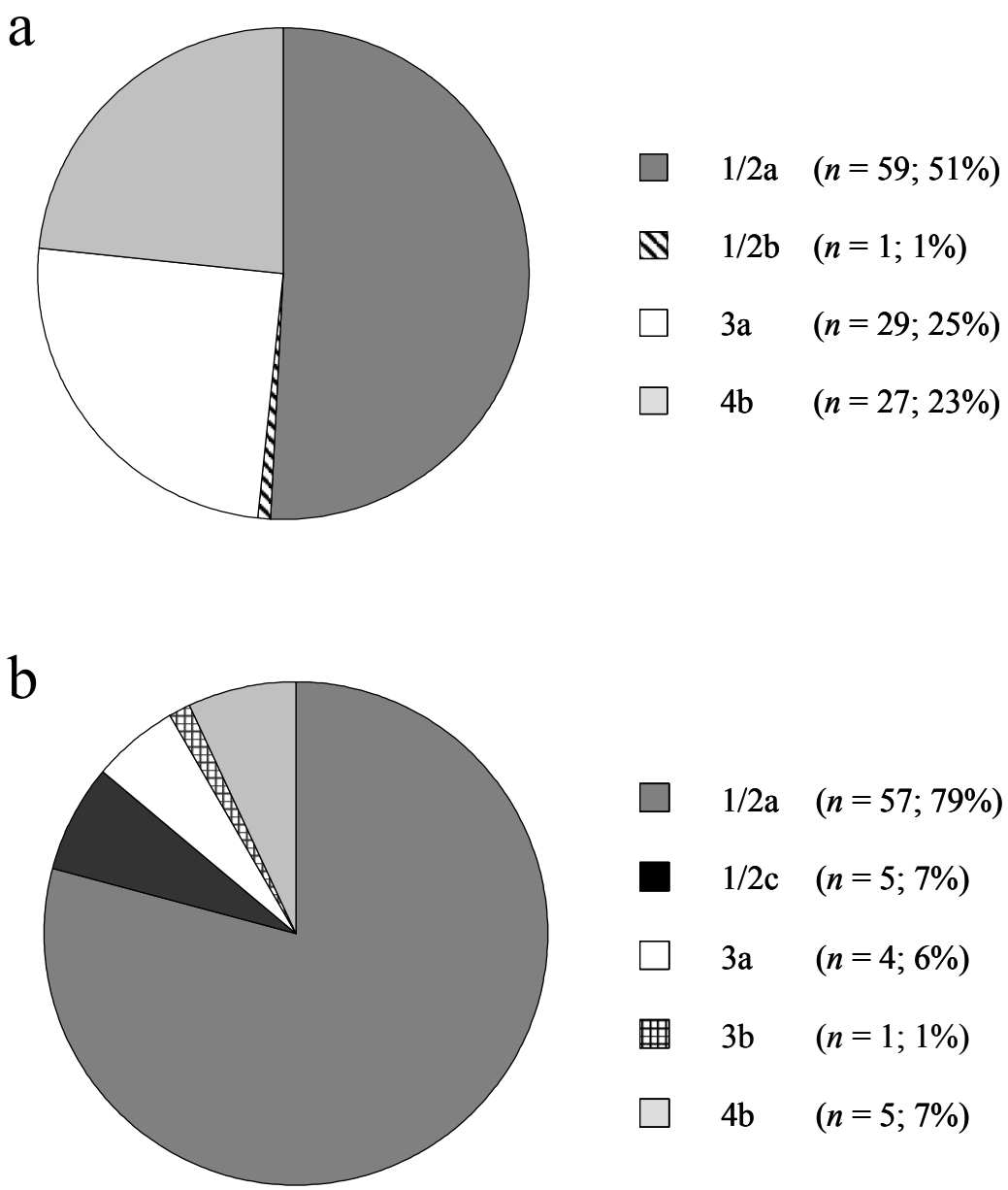
When the results of 188 strains isolated from humans (*n*=116) and the food industry (*n*=72) were compiled, automated ribotyping with restriction enzyme *Eco*RI divided the strains into 23 ribotypes (StudyV; Fig.1, Table 2 and 4). The dominating ribotypes were RT-1, RT-2 and RT-4. Two of these ribotypes (RT-1 and RT-4) contained isolates of two serotypes (1/2a, 1/2c and 1/2a, 3a respectively). PFGE divided these 188 strains into 54 PFGE types. When the results of sero-typing and ribotyping were combined with PFGE, 57 final subtypes were obtained. Ten (18%) of these 57 subtypes contained both human and food industrial isolates, 24 (42%) were found only among isolates from the food industry, and 23 (40%) among isolates from humans. Twenty-one food industrial isolates (26%, identified by V in study III) were never been identified among 314 human isolates in Finland.

Among strains isolated from 1997 to 1999, the discriminatory index (DI) for sero-typing, automated ribotyping and PFGE was 0.562, 0.873 and 0.946 respectively.

6. Electronic library

The electronic database library was built up using Bionumerics software. Thus far, when 383 (314 published in study V and 69 unpublished isolates) human *L. monocytogenes* isolates and 72 food industrial isolates have been analysed, the electronic database contains 128 PFGE profiles of *L. monocytogenes*: 107 PFGE profiles from human isolates and 21 (identified by V) from food industrial isolates. In establishing the library, any difference between two PFGE types was considered sufficient to distinguish two different PFGE types. The comparison of profiles has been faster and easier than before. In this study, the library has helped recognize the closely related strains (Study IV; Fig. 2).

Figure 8. Serotypes of 116 human (a) and 72 non-human (b) isolates from 1997 to 1999.



DISCUSSION

Food-borne diseases are a growing health problem worldwide and over 200 diseases are known to be transmitted by food (Bryan 1982). It has been estimated that food-borne diseases cause approximately 76 million cases, 325,000 hospitalisations and 5,000 deaths in the United States each year (Mead *et al.* 1999). In England and Wales in 2000, there were 1.3 million cases, 20,800 hospitalisations and 480 deaths due to indigenous food-borne diseases (Adak *et al.* 2002), and campylobacters, *C. perfringens*, salmonellas, EHEC O157 and *L. monocytogenes* accounted for the greatest disease burden. In Finland, it has been estimated that about half a million Finns become ill with food-borne diseases annually (Niemi *et al.* 1997). Several thousand individuals might later develop post infectious complications (Karmali *et al.* 1983; Keat 1983; Ropper 1992). However, only a fraction of all these food-borne infections can be traced to a certain vehicle and a specific causative agent. The traditional epidemiological microbiological methods such as biotyping, serotyping and phage typing of isolates, as well as the antimicrobial susceptibility testing, do not always give enough information for epidemiological purposes. Moreover, the final diagnosis might have been based mainly either on the clinical symptoms of the patients or findings in food. These background reasons lead us to start investigating the fingerprints of the outbreak and sporadic isolates of *S. enterica*, *C. perfringens* and *L. monocytogenes* by pheno- and genotyping methods. All these bacterial species are important food-borne pathogens from the public health point of view.

1. Methodological aspects

1.1. Diagnostics

C. perfringens strains causing food-borne diseases have a specific gene, the *cpe* gene which can be used to differentiate these strains from the strains of normal human faecal flora. The enterotoxin, encoded by this gene and produced in the small intestine, is responsible for the symptoms of food poisoning caused by these pathogenic strains. However, commensal *C. perfringens* strains are also usually found in human and animal stools, making the primary diagnostics difficult with the traditional culture methods. On the other hand, the diagnostics and interpretation of clinical significance is straightforward in salmonellosis since all *Salmonella* strains isolated from human specimens are considered significant findings even in asymptomatic subjects (Salyers and Whitt 1994). In addition, although some individuals (6%) may carry *L. monocytogenes* asymptomatically in their stools (Bojsen-Moller 1972; Muller 1990; MacGowan *et al.* 1991; Mascola *et al.* 1992; Schuchat *et al.* 1993; Slutsker and Schuchat 1999), this bacterium usually causes serious invasive infection and is, therefore, easily detected in microbiology laboratories from sterile body fluids of the patients.

1.2. Phenotyping *vs*. genotyping

The use of classical microbiological typing methods to investigate local outbreaks may be limited since some serotypes or phage types may predominate in a certain geographical area. For example, *S.* Enteritidis PT1 has been common in the Baltic countries and Russia (Hasenson *et al.* 1992), whereas PT4 is most often seen in the Western Europe

(Humphrey *et al.* 1989; Fantasia and Filetici 1994; Schroeter *et al.* 1994; Grimont *et al.* 1999; Tschape *et al.* 1999; Wall and Ward 1999; van de Giessen *et al.* 1999), Japan (Kusunoki *et al.* 1997) and Brazil (Irino *et al.* 1996) and PT8 and PT13a in the United States (Hickman-Brenner *et al.* 1991) and recently PT8 in Canada (Clark *et al.* 2003). Moreover, one fact which has to be considered when investigating the epidemiology of certain strains of *S*. Enteritidis by traditional phage typing is that some of them can change phage types (Threlfall and Chart 1993; Threlfall *et al.* 1993; Powell *et al.* 1995; Rankin and Platt 1995; Brown *et al.* 1999). These PT conversions have previously been shown to be related to loss of the ability to express long chain LPS, like the conversion of highly virulent strains of PT4 to strains of PT7 (Chart *et al.* 1989a) or related to plasmid acquisition, like the conversion of PT4 to PT24 (Frost *et al.* 1989). Phage conversions from PTs 1, 4, 8 to PTs 21, 6 and 13a (Brown *et al.* 1999) have also been reported. Furthermore, *L. monocytogenes* strains could mainly have been classified into three serotypes only, namely 1/2a, 1/2b and 4b (Farber and Peterkin 1991; Low *et al.* 1993). However, although the benefit of these traditional phenotypic methods is limited, they are very applicable screening methods when rapid preliminary differentiation of the strains is needed. This was seen, for example, in Finland when serotyping identified an infection outbreak caused by a rare *L. monocytogenes* serotype 3a in 1998 to 1999 (Lyytikäinen *et al.* 2000a). Moreover, in our study (I), PFGE alone without phage typing was not sufficient to distinguish between *S*. Enteritidis PT1 and PT4 since some strains showed identical PFGE banding patterns, regardless of their phage type. Also, Laconcha *et al.* (2000) pointed out that most of the 101 strains originating from three countries (Denmark, England and Spain) and belonging to PT1, PT4 or PT6, showed the same PFGE profile. Furthermore, Maslanka *et al.* (1999) suggested that PFGE provides a reliable method when used in conjunction with epidemiological data to diagnose *C. perfringens* food poisoning outbreaks. However, our study (II) clearly indicated that PFGE, in the absence of data on whether the isolates are enterotoxigenic or not, can yield misleading or even erroneous conclusions. For example, the investigation of isolates from the suspected outbreaks II and VII could have led to an incorrect finding that these outbreaks were not caused by *C. perfringens* since the isolates within these outbreaks had different PFGE types. PFGE is a very useful method in an outbreak in showing which isolates have identical macro-restriction profiles and, therefore, might be part of the same outbreak. However, this study on *C. perfringens* showed that before further laboratory typing, for example by PFGE, all pure cultures of *C. perfringens* isolates should be screened for the presence of the *cpe* gene and *S*. Enteritidis isolates should be phage typed.

Sporulation is considered necessary for *C. perfringens* enterotoxin expression. However, sporulation is often difficult to achieve for isolates grown in laboratory media (Harmon and Kautter 1986). Therefore, many laboratories have not routinely performed the detection of enterotoxin type A of *C. perfringens*. Also in this study, the determination of the enterotoxin production with two outbreak strains was difficult. The *cpe* –positive strain in one cluster remained CPE-negative and in the other the result with one *cpe*-positive strain remained uncertain twice and the third time only gave a CPE-positive result. Some *cpe*-positive strains may not be able to resist the heat treatment and, therefore, do not sporulate and produce a detectable amount of

enterotoxin. Another alternative is that the gene is a silent, unexpressed *cpe* gene. This is a disadvantage of PCR in that it can only identify isolates as potentially entero-toxigenic. An advantage of PCR over serologic assays is that it does not require the cells to sporulate *in vitro*.

Without analysing the *cpe* gene and without the information of the PFGE types of the other strains in those two clusters, the determination of CPE alone was inadequate in investigating those outbreak strains. Also Sparks *et al.* (2001) found one *cpe*-positive isolate which sporulated but did not express CPE. In a study of Kokai-Kun *et al.* (1994), 40% of the *cpe*-positive isolates from animals did not sporulate *in vitro* and were CPE-negative. These findings suggest the possibility of false negative results if only the determination of CPE is used and relied on.

The results of the antimicrobial susceptibility test are usually the first typing results available and might be the first sign of the outbreak if isolates from many individuals show an unusual or new pattern of antibiotic resistance. Also, long-term screening for antibiotic resistance gives an overview of the occurrence of resistance and information of the changes in the antimicrobial sensitivity patterns. However, because of many different genetic mechanisms, for example, acquisition or loss of plasmids carrying resistance genes, different strains can develop similar resistance patterns, thus, limiting the use of antimicrobial susceptibility testing. In this study, antimicrobial susceptibility testing was not useful with *S.* Enteritidis PT1 and PT4 strains because most of the strains were sensitive to all antimicrobial agents tested.

1.3. Interpretation of PFGE patterns

PFGE has been called “a golden method”. However, recent point mutation, deletion, insertion and losing or acquiring plasmids within a subtype might account for minor differences. These changes usually result in two to three fragment differences in PFGE banding patterns. Therefore, a generally accepted interpretation rule is that an isolate is closely related to an outbreak strain when the fragment difference is around two to three fragments, possible related when it is four to six and unrelated when the difference is seven or more (Tenover *et al.* 1995). In addition, when conclusions are drawn from genotypes, results should be linked to available epidemiological data. It has been shown that genotypically identical *L. monocytogenes* strains can be found from different food items, food processors and even in different countries (Autio *et al.* 2002). Therefore, identical PFGE profiles of the isolates from food and a patient do not prove, without epidemiological data, that the food isolate has caused the infection. However, depending on the bacterial strain, the PFGE method alone, even with epidemiological data, may not prove that this particular bacterium caused the outbreak. This was seen in the *C. perfringens* outbreaks in Finland, as mentioned above.

1.4. Issues concerning DNA degradation during PFGE

Some strains are not typeable by PFGE since the DNA of these strains is degraded during the electrophoresis. There are many reports of DNA degradation of different bacteria strains. For example, with strains of *Streptomyces lividans* (Ray *et al.* 1992), *Clostridium difficile* (Kristjansson *et al.* 1994; Corkill *et al.* 2000; Fawley and Wilcox 2002; Klaassen *et al.* 2002), *C. perfringens* (Ridell *et al.* 1998),

V. parahaemolyticus (Marshall *et al.* 1999), *Pseudomonas aeruginosa* (Römling and Tummler 2000), *S. enterica* and *E. coli* (Liesegang and Tschäpe 2002). In our laboratory, similar problems were previously faced with *S.* Newport strains (Lyytikäinen *et al.* 2000b) and with some *E. coli* strains (M. Eklund, personal communication). In study V, only one of nine *S.* Ohio strains was typeable while the rest degraded during the PFGE run. Therefore in this study, formaldehyde fixation, increased incubation times, varied concentrations of proteinase K and preincubation with lysozyme were used to try to avoid DNAase activity. However, none of these traditional changes in the method was useful in protecting the DNA from degradation.

Ray *et al.* (1992) demonstrated that during conventional electrophoresis site-specific cleavage of *S. lividans* DNA was Tris-dependent. In their study, the activation of reactive Tris radicals at the anode during the electrophoresis was prevented by adding 5 µM or more thiourea which react with the active component to the TBE. An alternative recommendation by Ray *et al.* (1992) was the use of another running buffer, HEPES, instead of the Tris-containing buffer TBE.

In the present study (V), HEPES running buffer was used because thiourea is a suspected carcinogenic agent. When Tris-containing TBE running buffer was changed to HEPES buffer, all previously untypeable *S.* Ohio and *S.* Newport (Lyytikäinen *et al.* 2000b) strains, as well as EHEC non-O157:H7 strains of serotypes O76:H19 and OX181:H49 (M. Eklund, personal communication), were typeable. The running voltage was reduced from 6 V/cm to 4 V/cm with HEPES, which contains a higher ionic strength than 0.5x TBE running buffer. However, later on it was noticed that the gel run with HEPES is comparable to gels run with TBE when the running voltage is reduced to 5 V/cm instead of to 4 V/cm (unpublished).

It seems that DNA degradation during electrophoresis is not a clonal trait because the only strain typeable with TBE running buffer shared the same *Xba*I and *Bln*I PFGE type with a strain which was affected by DNA degradation. This was also reported by Römling and Tummler (2000) who studied 1,200 strains of *P. aeruginosa* by PFGE. The DNA of 50 of these strains degraded. In a few cases, they found one isolate but not the others in a group of clonal variants affected by the degradation process.

1.5. Ribotyping *vs*. PFGE typing

Before a recently described standardized procedure which made it possible to complete the PFGE subtyping within 24 to 30 hours, the PFGE method was time consuming (Gautom 1997; Graves and Swaminathan 2001). However, it is also labour intensive and therefore, we evaluated the usefulness of automated ribotyping in comparison to PFGE. Like PFGE, the automated ribotyping is a very reproducible method and it is also rapid, simple to conduct, highly standardized and labour saving. In this study, the discrimination power of automated ribotyping among 188 *L. monocytogenes* isolates studied was lower (DI 0.873) than that of PFGE (DI 0.946). Some other authors also reported similar findings when enzymes *Eco*RI or *Pvu*II in manual ribotyping and *Apa*I or *Sma*I in PFGE were used (Louie *et al.* 1996; Kerouanton *et al.* 1998).

The WHO-sponsored international collaborative study reported that the discriminatory power of manual ribotyping with the *Eco*RI enzyme for serotype 4b of *L. monocytogenes* might be inadequate for epidemiological investigations (Swaminathan *et al.* 1996). Also in the

present study, the discriminatory index of ribotyping was slightly lower for *L. monocytogenes* serotype 4b (DI 0.762) than for the most common serotype 1/2a (DI 0.803). However, De Cesare *et al.* (2001a) have recently studied the suitability of 15 different enzymes for the discrimination of *L. monocytogenes* isolates in automated ribotyping and have shown that the use of different enzymes (*Pvu*II, *Eco*RI, *Xho*I) may significantly improve the discrimination among isolates of serotype 4b. The low DI power for serotype 3a, in the present study, is due to the same origin of most of the isolates.

Although, in general, PFGE in study III had higher DI than automated ribotyping, in three cases the PFGE type was divided into two different ribotypes. In these three cases, ribotyping was more discriminatory than PFGE. However, without serotyping, the ribotyping with *Eco*RI alone would not have been discriminative enough in investigating the outbreak caused by serotype 3a. These outbreak isolates of serotype 3a belonged to the ribotype RT-4 that also included nine isolates of other serotype (1/2a). Furthermore, the number of the PFGE types in study III was 54 and when the results of serotyping and ribotyping were compiled with PFGE, the number of subtypes only increased to 57. Therefore, ribotyping with enzyme *Eco*RI brought no extra discrimination to the results of PFGE in this study.

In study III, the *L. monocytogenes* isolates of one RT belonged to several PFGE types: the isolates within RT-1 were distributed into 11 PFGE types, RT-2 to 7 and RT-4 to 4. Among the isolates belonging to these RTs, the similarity values (calculated by Bionumerics software) of different PFGE types varied roughly from 50% to 93%, from 75% to100% and from 70% to 87%, respectively. The profiles of isolates differing by more than 7 fragments in PFGE are unrelated according to Tenover *et al.* (1995). Based on our experience, this corresponds to similarity values of about 70% or less. This indicates that some of the isolates in RT-1 and RT-4 were unrelated. Since any difference between two profiles was considered sufficient to distinguish two PFGE profiles, some of the isolates with these PFGE types were also closely related. In large ribogroups, including slightly different patterns of many isolates (same RTs) in the database, the tolerance to accept a new pattern is lower than in small ribogroups including only few patterns. For example, the ribogroup RT-1 already contains 286 patterns with a mean similarity value of 0.98 ±0.03. This suggests that automated ribotyping has limits in its applications, especially for closely related isolates and large ribogroups.

In study IV, some *L. monocytogenes* strains of different serotypes (1/2a and 3a) displayed indistinguishable PFGE profiles (2, 71, 207) and in study III different serotypes (1/2a, 1/2c and 1/2a, 3a) displayed indistinguishable ribotypes (RT1 and RT4, respectively). Ribotyping has also failed to classify strains of *L. monocytogenes* according to their serotypes in other studies (Louie *et al.* 1996; Kerouanton *et al.* 1998). However, in this study only one restriction enzyme, *Asc*I in PFGE and *Eco*RI in automated ribotyping was used; the use of a larger set of enzymes might have been more discriminatory for these strains. Furthermore, these serotypes (1/2a, 1/2c, 3a) have previously been grouped in the same genomic division by different genotypic methods (Brosch *et al.* 1994; Graves *et al.* 1994; Nadon *et al.* 2001). For example, the automated ribotyping divided 235 *L. monocytogenes* isolates into three lineages (I-III): lineage I included serotypes 1/2b, 3b, 3c and 4b; lineage II serotypes 1/2a, 1/2c and 3a; and lineage III serotypes 4a and 4c (Nadon *et al.* 2001). PFGE divided 176 *L. monocytogenes* isolates digested with *Asc*I into two divisions (I-II): division I included

serotypes 1/2a, 1/2c, 3a and 3c; and division II serotypes 1/2b, 3b, 4b, 4d and 4e (Brosch *et al.* 1994). PFGE with enzyme *Apa*I further divided isolates into cluster IA (serotypes 1/2c, 3c), cluster IB (serotypes 1/2a, 3a), cluster IIA (serotypes 1/2b, 3b) and cluster IIB (serotypes 4b, 4d, 4e).

1.6. Effects of plasmids and their usefulness in typing

Salmonella bacteria can spontaneously lose or acquire plasmids (Brown *et al.* 1991). This limits the application of plasmid analysis in epidemiological investigations. However, plasmid analysis was very useful when an outbreak of multi-resistant (R-type ACSSuSpT) *S.* Typhimurium DT 104 was investigated in England and Wales in 2000. While all 258 analysed isolates were identical by PFGE, 67% of them were characterized to be outbreak strains by harbouring a particular plasmid profile (Horby *et al.* 2003). The possible interference of unstable plasmids fragments is usually avoided by taking into account only fragments >125 kb (Olsen, J. E. *et al.* 1994), for example, when *S.* Enteritidis is analysed by PFGE. In the present study, both PFGE and plasmid profiling were capable of differentiating between strains of *S.* Enteritidis PT1 and between strains of PT4. Some studies have reported that PFGE identifies PT4 strains as members of one predominant clone, which has, therefore, limited the use of PFGE alone in differentiation of these strains (Desai *et al.* 2001; Liebana *et al.* 2001a). Also in the present study, some of the isolates of different PFGE subtypes had a difference of only one to two fragments between their PFGE banding patterns when digested with *Xba*I. Recent point mutations within a subtype may account for these minor differences (Tenover *et al.* 1995). However, the study of Murase *et al.* (1996) concluded that the PFGE profiles of strains digested with *Xba*I were not affected by point mutations. They subcultured nine isolates continuously for two days, and randomly selected colonies were digested by enzymes *Xba*I and *Bln*I. *Bln*I digested profiles were different from any of the profiles previously observed but there were no changes in *Xba*I digested profiles. In the present study, if there was a difference of several fragments between the plasmid profiles of two strains, it was considered to support the results of PFGE showing fragment patterns that differed by only a few fragments.

2. Microbiological tracing of *Salmonella* outbreaks

Finland has traditionally had a very strict salmonella control of production animals, which has made it easier to keep indigenously acquired salmonella infections at a low level. Therefore, most of the salmonella infections diagnosed in Finland have been associated with a trip abroad (Siitonen and Puohiniemi 1997; Siitonen 1998). In the early 1990s, *S.* Enteritidis caused 15 outbreaks in Finland; 12 of them were caused by strains of PT1 and PT4. However, it remained unclear from where these outbreaks originated. It was possible that the infections were connected to imported foodstuffs or that they were secondary infections caused by strains of foreign origin, or that there were some hidden reservoirs in Finnish production animals.

Poultry is one of most important reservoirs which can introduce *Salmonella* into the human food chain (Mishu *et al.* 1994; Schoeni *et al.* 1995; Kessel *et al.* 2001). The results of this study showed that the same *S.* Enteritidis PT1 1Fa strain had caused eight outbreaks in Turku from 1991 to 1995. Furthermore, this study confirmed the earlier findings (Johansson *et al.* 1996) that the outbreak strain indeed had originated from the nearby poultry farm. This was the first time when *S.* Enteritidis

PT1 was detected in a Finnish commercial layer flock (Johansson *et al.* 1996). The poultry farm was closed down in 1995, but how and from what source the farm had been contaminated by the PT1 strain was never discovered. There were some thoughts that this strain was somehow imported from the Baltic Countries where *S.* Enteritidis PT1 has been the most common *Salmonella* strain found in eggs (Hasenson *et al.* 1992). There are many investigations of the experimental studies where growth or penetration of PT4 but not PT1 strains have been studied in eggs and hens (Humphrey and Whitehead 1993; Schoeni *et al.* 1995; Fernandez *et al.* 2001). In the present study, the strains isolated in Estonia, and those isolated from the Finnish tourists who had visited the Baltic Countries, were of subtype 1Aa differing from the 1Fa outbreak strains by nine fragments. Therefore, according to "Tenover's rules" (Tenover *et al.* 1995) the strains were unrelated thus suggesting that the original source was not located in the Baltic Countries. Furthermore, the subtype 1Fa seemed to be very rare in Finland: only one single isolate among the 48 non-outbreak strains has belonged to this subtype that differed from the other non-outbreak subtypes by four to nine chromosomal fragments. The 1Fa strain was also found to be the cause of the outbreak in 1991 in Vieremä located about 500 km northeast of Turku. Unfortunately, no connection between the outbreaks caused by these identical strains in Vieremä and Turku could be found either at the time of the outbreak or later on.

There has been no identified reservoir for *S.* Enteritidis PT1 in Finland since the commercial layer flock outbreaks in 1991 to 1995. Therefore, it was an important finding that subtype 1Ba included the two isolates from Finnish cattle. An interesting finding was that among the subtype 1Aa, PT1 strains seemed to be associated with Eastern Europe and subtype 1Ba strains with Western Europe.

By the mid 1990s, *S.* Enteritidis PT 4 had caused three outbreaks in different parts of Finland: the first was in Leppävirta in 1993, the second in Rauma in March 1995 and the third in Kotka in December 1995. No connections were found between these three outbreaks at the time and their sources were never established. This *S.* Enteritidis PT 4 is the most common phage type that contaminates eggs in Central Europe (Schroeter *et al.* 1994) and in England (Humphrey *et al.* 1989). In Finland from 1995 to 1999, however, *S.* Enteritidis was very rarely found in cattle and turkeys, and never in eggs (except the PT1 outbreak strain in Turku).

In this study, most of the PT4 strains belonged to PFGE type 4A. However, plasmid profiling was able to distinguish six subtypes within this PFGE type. Among the 33 4A strains, only the outbreak strains in Leppävirta belonged to subtype 4Aa. Subtype 4Eb, which had caused the outbreak in Rauma, differed from 4Aa by three chromosomal fragments and three plasmid fragments, and was not found among other strains investigated here. The outbreak strain isolated in Kotka belonged to subtype 4Bg that differed from type 4Ab only by one plasmid (about 45-60 kb), which was about the same size as the fragment that distinguished subtypes B and A in PFGE. Thus, strains of subtypes 4Bg and 4Ab could have been of the same origin if the 4Bg strain had lost or 4Ab had acquired a plasmid of this size. This finding suggests that subtypes 4Bg and 4Ab, isolated from Finnish cows, potentially had their reservoir in Finnish cattle, and this reservoir might have been the source of the outbreak in Kotka if these strains had lost or acquired a plasmid. In addition, 4Bg and 4Eb differed by four chromosomal fragments, whereas 4Bg and 4Aa differed by only one fragment in PFGE. This one fragment was also less than 125 kb which could indicate interference from an unstable fragment of

plasmid DNA (Olsen, J. E. *et al.* 1994). However, these two types, 4Bg and 4Aa, differed by four plasmids. Considering all of these results, it seems that the outbreaks in Leppävirta, Rauma and Kotka had different origins.

3. Significance of faecal *C. perfringens* findings

In Finland, a standardized "faecal culture package" including cultures for *Salmonella, Shigella, Campylobacter, Yersinia, S. aureus, B. cereus* and *C. perfringens,* has been used to investigate food-borne outbreaks since the 1980s. However, when *C. perfringens* has been suspected to be the cause of an outbreak, the final diagnosis has mainly been based on the clinical symptoms of the patients and/or findings in foods, and the human faecal *C. perfringens* isolates have not commonly been studied further.

Forty-seven isolates related to nine food-borne outbreaks from 1984 to 1999 were retrospectively analysed by PCR for the *cpe* gene by RPLA to detect CPE enterotoxin production and by PFGE to compare the genotypes of the strains after restriction of their DNA by enzymes *Sma*I and *Apa*I.

All the strains available for further characterization were clearly part of the outbreak, namely they carried *cpe* and produced CPE, in only three of the nine suspected outbreaks. In four of the suspected clusters, both CPE- as well as *cpe*-positive and -negative strains were detected. In the two remaining suspected outbreaks, all isolates were CPE- and *cpe*-negative and had different PFGE subtypes, and therefore, these strains could not have caused these outbreaks. The *cpe*-negative strains found in these clusters probably were just members of the human normal flora. However, the clinical picture of the patients and the fact that no other food-borne bacterial pathogens were found from the faecal samples of these patients supported the hypothesis that *C. perfringens* was the causative agent. However, it cannot completely be excluded that the symptoms still were due to an agent not belonging to the diagnostic culture package. Our findings emphasize the importance of a more detailed characterization of *C. perfringens* isolates, rather than just identification to species level, in order to verify the bacterial cause of an outbreak.

The limitation of this study was the availability of only one pure culture of *C. perfringens* per patient. This culture might have represented a colony of the normal flora and might have been selected over enterotoxigenic *C. perfringens*. Van Damme-Jongsten *et al.* (1990) tested *C. perfringens* isolates from 186 unrelated food poisoning outbreaks. However, they tested also only one or occasionally two isolates from each outbreak. Only 60% of their isolates were positive for the *cpe* gene. It seems that they almost certainly characterized a number of *cpe*-negative *C. perfringens* of the normal flora - as we did in our study. Thus, it is very important to study more than one isolate from the faecal culture of the patient to increase the chance of finding significant *C. perfringens* strains, not only strains that are part of his or her normal flora. The recommendation to pick up 10 *C. perfringens* colonies per outbreak from faecal primary culture plates for *C. perfringens* has recently been introduced to the Finnish clinical microbiology laboratories (http://www.ktl.fi/ohjeita/vatsatautiepidemia, in Finnish).

The results of our study confirmed the earlier reports that the suspected food, meat casseroles, had caused the outbreaks in Helsinki in October 1997 and in March 1999 (Kukkula 1998; Hatakka and Halonen 2000). Meat and meat products have also been the most common vehicles

in outbreaks in many other countries (Todd 1997; Olsen, S. J. *et al.* 2000).

Only two of our 14 *cpe*-negative strains had identical PFGE profiles. Also, all *cpe*-negative strains, in clusters II, III, VII and IX, had different profiles (more than ten fragments) when compared to the *cpe*-positive strains. These findings are in contrast to the previous report stating that in a single outbreak the *cpe*-positive and -negative isolates have identical or nearly identical PFGE profiles (Ridell *et al.* 1998). In one cluster, the PFGE profiles (Ee and Ff) of two *cpe*-positive strains were different. This outbreak might have been caused by two different strains of *C. perfringens*. Maslanka *et al.* (1999) also obtained four unique PFGE patterns with 18 isolates from one outbreak. However, none of those isolates was tested for the ability to produce enterotoxin or for the presence of the *cpe* gene. Thus, it is unknown whether these strains were capable of producing symptoms of a food-borne disease or whether some of them just belonged to the normal flora.

4. Infection trends and clusters caused by *L. monocytogenes* in Finland

The 314 *L. monocytogenes* strains available for our study represented 75% of all 418 human listeriosis cases diagnosed from 1990 to 2001 (Siitonen 2003 and http://www.ktl.fi/ttr).

When the whole 11-year study period from 1990 to 2001 is concerned, the most common *L. monocytogenes* strains were of serotypes 1/2a and 4b, accounting, respectively, for 53% and 27% of the 314 cases of human listeriosis. After 1990, the percentage of annual cases caused by strains of serotype 4b has been fairly constant varying from 12 to 44%. However, the percentage of listeriosis cases caused by serotype 1/2a has increased and after 1990 it has been the most common serotype in Finland, varying from 35-67%. These results support the findings in the UK (McLauchlin and Newton 1995), Denmark (Gerner-Smidt *et al.* 1995), Switzerland (Pak *et al.* 2002) and Sweden (Loncarevic *et al.* 1998), suggesting that serotype 1/2a is replacing serotype 4b in human infections.

In France, the incidence (per million population) of *L. monocytogenes* infections has decreased by 68% from 1987 to 1997 (Goulet *et al.* 2001) and in the USA by 44% from 1989 to 1993 (Tappero *et al.* 1995). Also, in the UK the number of cases has decreased from 1983 to 1996 (Anonymous 1997). In Finland, the occurrence has been quite stable with an average 20 cases per year since 1990, except in 1997 (47 cases), 1998 (43 cases) and 1999 (45 cases). The outbreak affecting 25 people and caused by serotype 3a, PFGE type 71, explains in part the high number of cases in 1998 and 1999 (Lyytikäinen *et al.* 2000a). The findings of the present study also suggested that this outbreak had already started in 1997. According to our knowledge, this outbreak caused by the rare serotype 3a in Finland is unique.

The PFGE types containing at least five strains were compared with all other PFGE types in an established electronic database library (see Chapter 6) to see roughly if they were closely related to any other types according to the criteria of Tenover *et al.* (1995). These criteria are stringent and commonly appropriate for studies of strains collected over a short period. However, in the present study, grouping closely related types, collected over a long period, yielded more information on clusters. For example, PFGE type 1 was the most prevalent single type with 37 strains. It belonged to a major cluster, group G1, that represented 23% of all 314 strains studied. Furthermore, 27 strains of PFGE type 5 was the third most prevalent

single type, but it was not related to any other types. It also formed small clusters in 1997, 1998 and 2000.

PFGE type 1 has previously been associated with a vacuum-packed cold smoked rainbow trout product, and it caused febrile gastroenteritis in patients with no known underlying diseases in 1997 (Miettinen *et al.* 1999). In addition, Johansson *et al.* (1999) found isolates from retail ready-to-eat vacuum-packed fish products from four different producers in Finland in 1996 that were indistinguishable from PFGE type 1 (L. Rantala, personal communication) PFGE profile. However, when drawing conclusions from such genotyping data, results should be linked to available epidemiological data. Therefore, one of the problems in surveillance in listeriosis is the missing epidemiological information in sporadic cases. The PFGE type 1 associated with a vacuum-packed cold smoked rainbow trout product in 1997 (described above) was also common in sporadic cases in Finland from 1994 onwards, but not earlier. This increased prevalence of infections caused by strains of PFGE type 1 could reflect a change in dietary habits, that is vacuum-packed fish products might be more favoured in diets or this PFGE type might be more pathogenic than other PFGE types, or this type is simply commoner than others in fish products.

No specific serotype or PFGE type can be concluded to be associated with pregnancy cases, any particular age group or gender. However, the only exception was the PFGE type 24, which was statistically significantly associated with gender; as far as we are aware this finding has not been reported earlier.

5. Diversity of human and non human *L. monocytogenes* isolates

We also ribotyped 116 *L. monocytogenes* strains isolated during a three-year period (1997 to1999) and representing a subset of the human strains described above. All their typing results (O:H serotype, PFGE and ribotype) were compared to those of 72 *L. monocytogenes* isolates from the food processing industry from the same time period and analysed in an identical way. According to these results, only 7 % of the food industry isolates, but 23% of the human isolates, belonged to the serotype 4b. This supports the earlier findings that although serotype 4b has caused a number of outbreaks, it has been isolated more rarely from foodstuffs which are more commonly contaminated by serotype 1/2 isolates (Rocourt 1988; Dauphin *et al.* 2001). In a recent study, Buncic *et al.* (2001) found that the 1/2a isolates tended to be more resistant than the 4b isolates to two tested antilisterial bacteriocins at 4°C. This finding may partly explain why strains of serotype 1/2a have been isolated from foodstuffs more often than 4b in industrial processing. However, they also reported that after cold storage at 4°C, 4b isolates tended to be more resistant to heat treatment at 60°C and had higher pathogenicity than 1/2a isolates when transferred from cold storage to body temperature. This finding may explain that the strains of serotype 4b have caused more outbreaks than the strains of serotype 1/2a.

Furthermore, only 18% of the final subtypes (combination of the results of serotyping, riboryping and PFGE) included both human and food industrial isolates although the isolates were from the same time period, 1997 to 1999. However, two final subtypes (1 and 9), in particular, dominated among humans, and identical subtypes were also found from food processing plants in Finland. In addition,

surprisingly many (26%) of the PFGE types (Study III, identified by V) originating from Finnish food processing plants were never (from 1990 to 2001) identified among human isolates in Finland. In the future, it would be very interesting to compare the potential pathogenicity between isolates often isolated from humans' invasive infections and isolates never identified among human isolates. Larsen *et al.* (2002) investigated seven different PFGE types of *L. monocytogenes* originated from human clinical cases and food, showing a significant correlation between PFGE type and their ability to invade Caco-2 cells. The study also supported the hypothesis that certain PFGE types of *L. monocytogenes* commonly found in food are less invasive than others to Caco-2 cells (Larsen *et al.* 2002).

6. Electronic library – empowerment in surveillance

A database library containing all different PFGE profiles of *L. monocytogenes* strains studied was established using Bionumerics software. Any difference between two PFGE types was considered sufficient to distinguish two different PFGE types. In this new electronic library, the PFGE profiles can be compared with each other more rapidly than by just naked eye and, therefore, clusters can also be detected more rapidly and at an early stage. This library also empowers the continuous surveillance of serious *L. monocytogenes* infections in Finland. When the library was established, a bilateral computer-based network for the comparison of PFGE profiles of *L. monocytogenes* isolates from humans, food and food production environments was begun (Rantala *et al.* 2001) with The National Veterinary and Food Research Institute (EELA). Such national cooperation during a suspected outbreak will help in recognizing the sources of infections. It will also enable the authorities to track down the food production plants that need to improve their food production hygiene. During the 1990s, clusters of cases of the same PFGE type or clusters of groups of closely related types were seen every year (as discussed in Chapter 4) but noticed only during this study. These findings clearly showed the need for and the importance of thorough typing of human *L. monocytogenes* strains and the timely comparison of the typing results. An electronic network of PFGE profiles of human *L. monocytogenes* strains, has been in use as PulseNet since 1996 in the USA (Swaminathan *et al.* 2001), where it has demonstrated its value in early recognition of outbreaks and rapid identification of their sources.

Finland has also participated in an international genotyping project (Salmgene) which is a European collaboration for harmonizing DNA fingerprinting for food-borne *Salmonella* strains (Peters *et al.* 2003). Free movement of people and goods between countries is creating a need for such international surveillance. During this collaboration, the PFGE protocol has been standardized between participating countries (Peters *et al.* 2003) and a large collection of *Salmonella* strains with their pheno-and genotyping results and with epidemiological data will be created as an electronic format. This international database of PFGE profiles will make the exchange of molecular information between different countries in the EU more effective.

CONCLUSIONS

In this thesis, phenotypic and molecular methods were used to gain detailed information of the diversity of the food-borne *S. enterica*, *L. monocytogenes* and *C. perfringens*. These bacteria are important from the public health point of view causing small clusters or larger outbreaks annually. Also, the virulence-associated characteristic of *C. perfringens* was investigated to improve the differential diagnostics of this bacterium.

PFGE was a very useful and key method for typing and grouping these food-borne bacteria and was very suitable for epidemiological purposes when bacterial strains isolated from outbreaks and sporadic cases were differentiated. It was also essential to complement the results of the phenotyping methods. However, PFGE was unable to replace conventional and internationally standardised phenotypic methods. Namely, the findings in this thesis showed that in the absence of certain phenotypic and epidemiological data, PFGE alone did not distinguish between strains of *S.* Enteritidis PT1 and PT4 or between pathogenic and commensal strains of *C. perfringens*. Therefore, before typing by PFGE, *S.* Enteritidis isolates should be phage typed and all *C. perfringens* isolates screened for the *cpe* gene.

The conditions for preventing the DNA degradation during PFGE were found. When Tris-containing running buffer was changed to HEPES buffer, all previously untypeable *S.* Ohio, *S.* Newport and EHEC non-O157 strains were typeable. This positive effect of HEPES buffer in PFGE was an important application because: i) PFGE has become one of the most exploited genotyping method, ii) in contrast to HEPES, thiourea, that previously has been used to prevent the DNA degradation, is classified as a cancer-causing agent.

The discrimination power of automated ribotyping among *L. monocytogenes* isolates was lower than that of PFGE and ribotyping with enzyme *Eco*RI brought no extra discrimination to the results of PFGE. Furthermore, without serotyping the ribotyping with *Eco*RI alone was not discriminative enough in investigating the outbreak caused by a strain of the serotype 3a. In addition, the automated ribotyping has limited applications especially for closely related isolates and larger ribogroups. However, mainly based on the ability of the automated ribotyping to analyse a large number of bacterial isolates in a very short time with minimal human labour, it is a very useful tool as the first step method in epidemiological surveys.

The genotyping results now confirmed the earlier findings that the *S.* Enteritidis PT1 strain, which had caused eight outbreaks in the surroundings of Turku from 1991 to 1995, indeed originated from the nearby poultry farm. An identical strain was also found to be the cause of an outbreak in Vieremä, far from Turku. Further in-vestigations showed that the PT1 isolates associating with these outbreaks, differed from those PT1 isolates predominating in the Baltic Countries and Russia. It was also established that the three outbreaks caused by *S.* Enteritidis PT4 all had different origins. Furthermore, valuable information for future investigations was gained on the distribution of molecular subtypes of the strains that were imported from the tourist resorts popular among Finns, as well as on the subtypes of those strains that were isolated from domestic production animals.

The true outbreaks caused by *C. perfringens* in Finland were detected and confirmed by assaying the *cpe* gene and CPE production. These results were subsequently supported by the results of the PFGE profiles of the strains. However,

in two infection clusters the strains were, contrary to previous suspicions, not the causative agents of the food poisonings. Thus, the results clearly emphasized the importance of the characterization of the *C. perfringens* isolates in more detail than just identification to the species level in order to verify the cause of an outbreak. The PFGE typing or determination of production of CPE enterotoxin alone may even lead to the false interpretation of the causative agent. Therefore, the determination of the *cpe* gene by PCR was found to be crucial. It was also found that it is very important to study more than one isolate from the faecal culture of the patient to increase the probability of detecting the cells carrying the *cpe* gene and not just cells being part of the normal flora. Consequently, the recommendation to pick up 10 *C. perfringens* colonies per outbreak for further testing has recently been introduced to the Finnish clinical microbiology laboratories.

L. monocytogenes serotypes 1/2a and 4b predominated among strains isolated from human infections in Finland from 1990 to 2001. The data suggested that the strains of the serotype 1/2a have been replacing those of the serotype 4b. Much information was gained on the prevalence of different serotypes among human and food industry strains. The most prevalent single genotype was PFGE type 1; this type has previously been associated with vacuum-packed fish products. However, one problem with the sporadic listeriosis cases was the missing epidemiological data making it difficult to draw conclusions. The findings also suggested that the outbreak that was associated with butter and was caused by the strain of the serotype 3a, had already started in 1997, not in 1998 as reported earlier. Other interesting findings were also that PFGE type 24 was statistically significantly associated with the male gender and more than one fourth of the industrial isolates were never detected as causing human infections.

An electronic database library of the different PFGE profiles of human *L. monocytogenes* strains was created. This library has already been very valuable in continuous and real-time surveillance of serious *L. monocytogenes* infections in Finland making the detection of infection clusters possible and easy. At present, the library is in “everyday use” in bilateral computer-based networking for the comparison of PFGE profiles of *L. monocytogenes* isolates from humans, food and food production environments in collaboration with The National Veterinary and Food Research Institute (EELA).

As a result of this work, the identification of three important food-borne bacterial pathogens has become more specific and reliable in epidemiological surveys. In the future, new typing techniques like DNA microarrays and other methods exploiting the availability of full-genome sequences will open new visions of microbial pathogenity and help us to understand better the origin and spread of microbial diseases. Furthermore, the close co-operation at the local, national and international level with the authorities responsible for surveillance and preventing of food-borne diseases should not be forgotten.

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